

Watching bacteriorhodopsin
pump protons *p. 1552*

Boron nitride paves a
path to propylene *p. 1570*

Trillions of insects migrate
over our heads *p. 1584*

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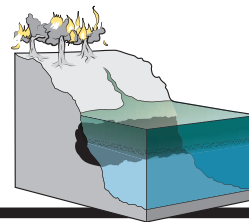
2016

BREAKTHROUGH
of the **YEAR**



CONTENTS

23 DECEMBER 2016 • VOLUME 354 • ISSUE 6319



1529

A deadly oxygen-phosphorus cycle

SPECIAL SECTION

Breakthrough of the Year

WINNER

1516 The cosmos aquiver

1517 People's choice

RUNNERS-UP

1518 The exoplanet next door

1518 Artificial intelligence ups its game

1519 Killing old cells to stay young

1520 Mind-reading great apes

1520 Proteins by design

1521 Mouse eggs made in the lab

1522 A single wave of migration from Africa peopled the globe

1522 Genome sequencing in the hand and bush

1523 Metalenses, megapromise

OTHER FEATURES

1523 Scorecard for 2016

1524 Areas to watch in 2017

1525 Breakdowns of the year

SEE ALSO

► PODCAST; VIDEO; QUIZ; EDITORIAL P. 1507

ON THE COVER



Star trails arc above a 4-kilometer-long arm of the Laser Interferometer Gravitational-Wave Observatory (LIGO) detector in Hanford, Washington (for more on the photograph,

see <http://scim.ag/2gGRzG7>). Early this year, LIGO scientists announced that their two detectors—the second is in Livingston, Louisiana—had recorded a minuscule quiver in spacetime, generated by black holes colliding in the distant universe. The feat, *Science's* 2016 Breakthrough of the Year, confirmed a century-old prediction by Albert Einstein and opened a new window on the universe. See page 1516.

Photo: © Rich Frishman

NEWS

IN BRIEF

1508 News at a glance

IN DEPTH

1510 XPRIZE FINALISTS MULL PAYLOADS TO THE MOON

Science experiments take a back seat to commercial aims for five teams planning launches *By D. Clery*

1511 IN INDIA, ELITE INSTITUTES IN SHADY JOURNALS

Predatory publishers draw papers from faculty at top research centers *By P. Pulla*

1512 NEW BLOOD TESTS MAKE STRIDES IN DETECTING PRION DISEASE

Long after the European mad cow outbreak, researchers hope to screen blood supplies for misfolded proteins *By K. Servick*

► 10.1126/scitranslmed.aaf6188;

10.1126/scitranslmed.aag1257

1513 SCIENTISTS FLAG NEW CAUSES FOR SURGE IN METHANE LEVELS

Swelling tropical wetlands and a decline in a natural atmospheric detergent are leading suspects *By P. Voosen*

1514 CRITICS ASSAIL PAPER CLAIMING HARM FROM CANCER VACCINE

Vaccination rates fall as antivax campaigns gather steam *By D. Normile*

1515 LIKE BIRDS, INSECTS MAY TRAVEL IN SYNC WITH THE SEASONS

Long-term radar studies reveal masses of winged migrants *By E. Pennisi*

► REPORT P. 1584

INSIGHTS

POLICY FORUM

1526 EXPAND INNOVATION FINANCE VIA CROWDFUNDING

Crowdfunding attracts venture capital to new regions *By O. Sorenson et al.*

1515 & 1584



PERSPECTIVES

1529 OCEANS ON THE EDGE OF ANOXIA

Environmental crises can tip the ocean into O₂ depletion *By A. J. Watson*

1530 A STITCH IN TIME SAVES NINE...BILLION

Acting now to limit global temperature change will help to ensure global food security *By E. A. Fulton*

► REPORT P. 1591

1532 OPTICAL CIRCULATORS REACH THE QUANTUM LEVEL

The spin state of a single atom can route light signals in different directions *By W. J. Munro and K. Nemoto*

► REPORT P. 1577

1533 THE "TAO" OF INTEGUMENTS

Hair follicle and sweat gland fates can be switched by morphogens at specific skin regions or developmental stages *By Y. C. Lai and C.-M. Chuong*

► REPORT P. 1551

1534 "PHENO" MENAL VALUE FOR HUMAN HEALTH

Rare genetic variants are linked to electronic health record phenotypes at a population scale *By D. J. Rader and S. M. Damrauer*

► RESEARCH ARTICLES PP. 1549 & 1550

1536 MORE THAN A DAY IN THE LIFE OF A COMET

Learning how a comet evolves can reveal the nature of the early solar system *By N. Dello Russo*

► REPORTS PP. 1563 & 1566

CONTENTS



1533 & 1551

The yin and yang
of hair and sweat

23 DECEMBER 2016 • VOLUME 354 • ISSUE 6319

BOOKS ET AL.

1538 TOMORROW'S ARSENAL

Two authors probe the technologies transforming warfare *By N. Al-Rodhan*

1539 DIVINING SCIENCE

A new tome argues that the ancient Babylonians deserve greater recognition in scientific histories *By A. Robinson*

LETTERS

1541 FOREST VALUE: MORE THAN COMMERCIAL

By C. Paul and T. Knoke

1541 RESPONSE

By C. B. Barrett et al.

1542 IRAN'S SCIENCE LANDSCAPE IN CONTEXT

By R. Mansouri

1543 TECHNICAL COMMENT ABSTRACTS

1543 ERRATA

RESEARCH

IN BRIEF

1546 From *Science* and other journals

RESEARCH ARTICLES

HUMAN GENETICS

1549 Distribution and clinical impact of functional variants in 50,726 whole-exome sequences from the DiscovEHR Study *F. E. Dewey et al.*
RESEARCH ARTICLE SUMMARY; FOR FULL TEXT: [dx.doi.org/10.1126/science.aaf6814](https://doi.org/10.1126/science.aaf6814)

1550 Genetic identification of familial hypercholesterolemia within a single U.S. health care system *N. S. Abul-Husn et al.*
RESEARCH ARTICLE SUMMARY; FOR FULL TEXT: [dx.doi.org/10.1126/science.aaf7000](https://doi.org/10.1126/science.aaf7000)
► PERSPECTIVE P. 1534

1551 CELL FATE DECISIONS

Spatiotemporal antagonism in mesenchymal-epithelial signaling in sweat versus hair fate decision *C. P. Lu et al.*
RESEARCH ARTICLE SUMMARY; FOR FULL TEXT: [dx.doi.org/10.1126/science.aah6102](https://doi.org/10.1126/science.aah6102)
► PERSPECTIVE P. 1533

1552 STRUCTURAL BIOLOGY

A three-dimensional movie of structural changes in bacteriorhodopsin *E. Nango et al.*

1557 TOPOLOGICAL MATTER

Majorana bound state in a coupled quantum-dot hybrid-nanowire system *M. T. Deng et al.*

REPORTS

COMETARY SCIENCE

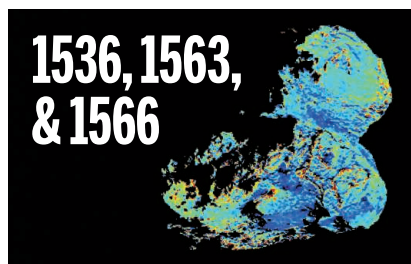
1563 Seasonal exposure of carbon dioxide ice on the nucleus of comet 67P/Churyumov-Gerasimenko *G. Filacchione et al.*
1566 Rosetta's comet 67P/Churyumov-Gerasimenko sheds its dusty mantle to reveal its icy nature *S. Fornasier et al.*
► PERSPECTIVE P. 1536

1570 CATALYSIS

Selective oxidative dehydrogenation of propane to propene using boron nitride catalysts *J. T. Grant et al.*

1573 QUANTUM ELECTRONICS

Suppressing relaxation in superconducting qubits by quasiparticle pumping *S. Gustavsson et al.*



1577 QUANTUM OPTICS

Quantum optical circulator controlled by a single chirally coupled atom *M. Scheucher et al.*
► PERSPECTIVE P. 1532

1580 NANOMATERIALS

Emergence of hierarchical structural complexities in nanoparticles and their assembly *C. Zeng et al.*

1584 MIGRATION

Mass seasonal bioflows of high-flying insect migrants *G. Hu et al.*
► NEWS STORY P. 1515; VIDEO

1587 BRAIN RESEARCH

Active cortical dendrites modulate perception *N. Takahashi et al.*

1591 FISHERIES

Large benefits to marine fisheries of meeting the 1.5°C global warming target *W. W. L. Cheung et al.*
► PERSPECTIVE P. 1530

1594 PLANT SCIENCE

Precursor processing for plant peptide hormone maturation by subtilisin-like serine proteinases *K. Schardon et al.*

1597 STRUCTURAL BIOLOGY

Crystal structure of unlinked NS2B-NS3 protease from Zika virus *Z. Zhang et al.*

DEPARTMENTS

1507 EDITORIAL

Awesome universal chirp *By Jeremy Berg*
► BREAKTHROUGH OF THE YEAR SECTION P. 1516

1666 WORKING LIFE

Rescuing my time from science *By Luca Rinaldi*

Science Staff1504
AAAS News & Notes1544
New Products1601
Science Careers1602

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Awesome universal chirp

In 1978, while I was an undergraduate student, Kip Thorne came to Stanford University to give a seminar on a proposed search for gravitational waves. The auditorium quickly filled with experts and inquisitive students like me. As Thorne described a quest for these ripples in space-time, the audience grew palpably skeptical. Albert Einstein had predicted the existence of gravitational waves based on his theory of general relativity, but even he was doubtful that they would ever be detected because they were anticipated to be so weak. Listening to Thorne propose an approach for measuring such waves, I had no idea that he and his colleagues were about to begin a 40-year journey to discover what *Science* now recognizes as the 2016 Breakthrough of the Year. The announcement that gravitational waves had indeed been detected opens up a new window into the universe (see the News story on p. 1516).

The planning and experimentation that led to the Laser Interferometer Gravitational-Wave Observatory (LIGO), the apparatus that detected the gravitational waves, illustrate how the interplay between theory and experiment can provide the impetus for building remarkable new tools. LIGO depends on measuring changes in length that amount to a small fraction of the diameter of an atomic nucleus over a distance of several kilometers. After many years of construction, and just after the completion of a substantial upgrade, LIGO observatories situated in Hanford, Washington, and Livingston, Louisiana, detected a “chirp” lasting about a tenth of a second on 14 September 2015. The detailed features of this chirp could be fit by Einstein’s equations with impressive accuracy. The original paper describing this discovery is a joy to read,* even without understanding the technical details. Two black holes, each with about 30 solar masses, circled one another and then coalesced into a single black hole. In this process, the energy associated with these three masses was released in the form of gravitational waves. Although this discovery is a singular event, its importance lies in

the observations yet to come. An additional, but smaller, black hole merger has already been observed, and more are expected, particularly with the sophisticated detectors that are in development. More measurements of events across the space and time of the universe may lead to theories and observations of other phenomena that will need explanation.

Science’s runners-up include many exciting discoveries that can be divided (somewhat arbitrarily) into two categories.

Some breakthroughs were primarily technological advances, spanning a range of fields. These include remarkable progress in artificial intelligence; DNA sequencers based on passing DNA strands through nanopores; coaxing stem cells to form eggs capable of producing viable mammals; the construction of novel small-scale lenses; and programs that can design proteins and protein assemblies with predetermined structures. This category also contains *Science*’s People’s Choice for the Breakthrough of the Year—the ability to grow human embryos in culture for nearly 2 weeks, much longer than had been achieved previously, opening up new research opportunities but also

raising important ethical questions.

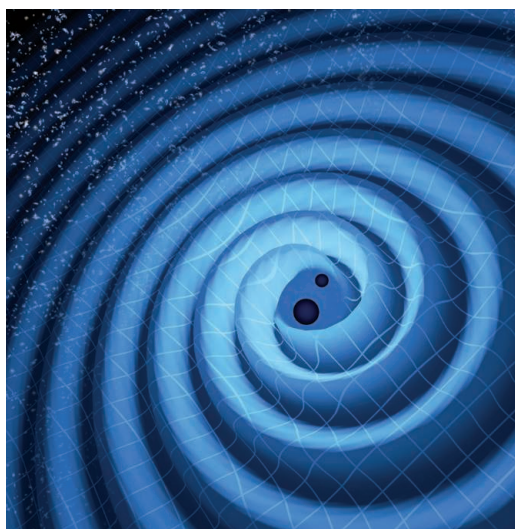
Other breakthroughs represent more conceptual advances. These include the discovery of an exoplanet only 4 light-years from Earth; a genomic analysis that supports the notion that all modern human beings derive from a single migration out of Africa; new insights into the similarities between human and ape minds; and the demonstration that clearing mice of senescent cells can slow the aging process.

Selection of the Breakthrough of the Year should be viewed not only as a competition for top positions, but as a celebration of the remarkable scientific advances that are published over the course of a year. It is a pleasure to compile and think through all of the exciting results that have been published, and a challenge to winnow the field down to a final list.

—Jeremy Berg



Editor-in-Chief,
Science Journals.
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**“...even [Einstein] was doubtful
that they would ever be detected...”**

*B. P. Abbott et al., *Phys. Rev. Lett.* **116**, 061102 (2016).

Amount raised at auction for a rare first edition of Isaac Newton's *Philosophiæ Naturalis Principia Mathematica*, which was published in 1687 and sets out Newton's laws of motion. Purchased anonymously, it is now the most expensive science book ever sold.

IN BRIEF

Volcanic carbon dioxide seen from space



A NASA orbiting observatory last year detected Mount Yasur's carbon dioxide plume.

The reliably active and tourist-accessible Mount Yasur, a volcano on the remote Pacific archipelago of Vanuatu, has long been a local attraction. But now, it could become an international landmark. Yasur (shown in 2010) may be the first volcano to burp up a plume of carbon dioxide (CO_2) measured from space. In May 2015, researchers using NASA's Orbiting Carbon Observatory-2 (OCO-2) found that the volcano was belching CO_2 into the air at a rate of 42 kilotons per day. That was a surprise: The team never expected to record the emissions of individual point sources of CO_2 , like volcanoes or coal-fired power plants. Although volcanic burps of sulfur dioxide stand out against trace background levels, the atmosphere has higher background levels of CO_2 ; moreover, winds disperse each injection quickly, and clouds, dust, and distance can all confound the results, the team said 14 December at the annual meeting of the American Geophysical Union in San Francisco, California. But OCO-2 happened to pass directly above Yasur when its emissions were popping against a background of pristine Pacific air. The lucky detection points the way toward viable verification of greenhouse gas emissions, needed to help enforce climate treaties—and foreshadows space-based monitoring of the fumes that herald volcanic eruptions.

AROUND THE WORLD

New rule shields U.S. streams

WASHINGTON, D.C. | U.S. President Barack Obama's administration on 19 December finalized a controversial—and long-awaited—rule designed to protect small streams and forests from coal mining. The so-called Stream Protection Rule has been nearly 8 years in the making, and included substantial input from hydrologists, stream ecologists, and water quality researchers. The rule will require firms to make greater efforts to forecast and monitor the effects of mining on nearby waterways and forests, and the Department of the Interior predicts it will protect nearly 10,000 kilometers of streams and 21,000 hectares of forest over the next 2 decades. But the mining industry and some Republican lawmakers oppose the rule, arguing it imposes burdensome costs on an already struggling industry, and have promised to undo it after President-elect Donald Trump takes office in January 2017.

OK on mitochondrial replacement

LONDON | Authorities in the United Kingdom have given the go-ahead for mitochondrial replacement therapy, a new type of assisted reproduction that could allow some families to avoid passing on genetic disease. The widely anticipated decision, announced 15 December by the Human Fertilisation and Embryology Authority, allows clinics in the United Kingdom to apply for a license to treat patients with the controversial technique, which combines genetic material from three people: two eggs and one sperm. Mitochondrial



New technique circumvents diseases of mitochondria.



Glowworms illuminate limestone caves in New Zealand.

Glowworms keep their fishing lines sticky with a surprising ingredient

Glowworms are the source of the eerie blue lights that dazzle hundreds of thousands of visitors to New Zealand's Waitomo Caves each year. But when it comes to how they eat, the famous worms still hold some mystery: Specifically, they use a surprising ingredient to help trap their prey. The worms are the larvae of a species of fungus gnats endemic to the country; their posteriors glow because of a chemical reaction in their Malpighian tubules—structures analogous to kidneys. To eat, they dangle

"fishing lines" of mucus and silk, secreted from glands in their mouths and dotted with sticky droplets. It's a strategy apparently similar to that of spiders weaving their webs, but whereas spider silk is a complex mixture of silk and glycoproteins, the sticky droplets on glowworm lures are 99% water—with a dash of urea, the main component of urine, scientists reported last week in *PLOS ONE*. How the mouth-secreted mucus threads come to have small amounts of urea—made at the other end of the body—is a topic for future study.

disease, which can include symptoms from brain damage to heart problems, results from mutations in the DNA carried in a person's mitochondria, cellular energy sources that carry their own set of genes. Women who carry the mutations can pass them on to their children. Researchers have developed several methods that involve combining the nucleus from an affected woman's egg—which contains the bulk of the DNA—with an unaffected woman's cytoplasm, which contains the mitochondria. At least one baby has been born after the procedure, used by doctors working in Mexico. But recent work has found that some of the defective mitochondria from the mother's egg can make it into the embryo along with the nuclear DNA.

Innovation bill squeaks through

WASHINGTON, D.C. | A once-controversial bill to bolster innovation and research activities at the National Science Foundation (NSF), the National Institute of Standards and Technology, and various research and education programs managed by the White House Office of Science and Technology Policy (*Science*, 9 December,

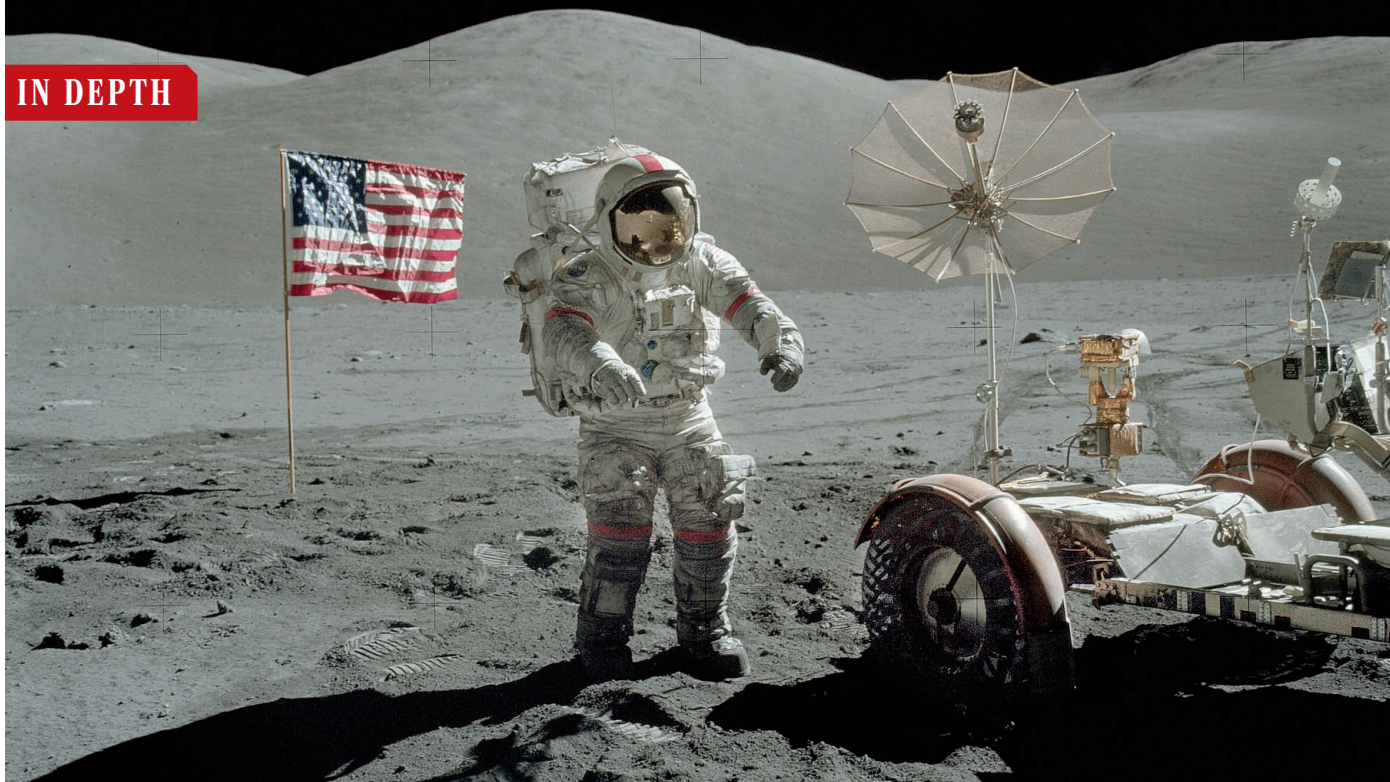
p. 1209) will soon become law after the U.S. House of Representatives unexpectedly cleared it last week. On 12 December the Senate had passed a compromise version of S. 3084 that backed away from provisions in various House bills affecting how NSF manages its research portfolio. Academic scientists had strongly opposed them, and they had triggered a bitter, 3-year partisan battle in Congress. House members then left for the holidays, and the bill appeared dead. However, the bill's proponents used a parliamentary loophole through which unoffending legislation can be approved if no member objects. On 16 December Representative Barry Loudermilk (R-GA) introduced the measure and, seconds later, it was deemed passed by unanimous consent. The American Innovation and Competitiveness Act will replace a 2010 law covering programs at those agencies that expired in 2013.

FINDING

Pregnancy reshapes the brain

The architecture of women's brains changes strikingly during their first

pregnancies in ways that last for at least 2 years, scientists report this week in *Nature Neuroscience*. Animal studies have documented many neural changes, but virtually nothing was known about humans. The team used MRI to scan the brains of 25 women before their first pregnancies and then several weeks after they gave birth. They also scanned first-time fathers, childless men, and childless women. Then, they used computer-based analyses to measure changes in gray matter volume. Unlike the others, the mothers showed highly consistent losses, particularly in areas that process and respond to social signals. The size of the changes significantly predicted the quality of the new mothers' bonding with their infants. This may mean that pregnant women's brains adapt in areas that allow them, for instance, to better respond to their infants' needs or to detect threatening people. Two years later, MRI scans of 11 of the 25 mothers—those who had not become pregnant again—showed that gray matter loss remained. But in the hippocampus, a region associated with memory, most volume was restored. <http://scim.ag/prebrains>



PLANETARY SCIENCE

XPrize finalists mull payloads to the moon

Science experiments take a back seat to commercial aims for five teams planning launches

By Daniel Clery

A few years back, Oded Aharonson, a planetary scientist at the Weizmann Institute of Science in Rehovot, Israel, met three space-mad engineers who were building a cut-price mission to the moon. Backed by a mix of companies, foundations, and universities, the trio was competing for the \$20 million jackpot of the Google Lunar XPrize, which challenged privately funded teams to be the first to land on the moon, travel 500 meters, and send back pictures and video. Other than that, the engineers' ambitions didn't extend beyond a triumphant party in the central square of Tel Aviv, Israel.

But to Aharonson, this was too good an opportunity to miss. "You have to do something more. There must be some intellectual legacy to this mission," he told them. After several such conversations, he says, "they bought it." The party plans are still on. But SpaceIL now has a mission scientist—Aharonson—and its lander will carry a lightweight sensor to map the moon's magnetic field.

Science was never the primary driver for

the Lunar XPrize, which reaches a major milestone at the end of this month. Only those teams with a contract to launch their spacecraft before the end of 2017 will be allowed to stay in the competition. Of the 16 industry teams still in the running, the XPrize authorities have confirmed launches for just four, including SpaceIL. A fifth team, Part-Time Scientists, a Germany-based team founded by researchers who initially entered the prize alongside their day jobs, is still awaiting confirmation of its launch booking.

Engineering will determine the ultimate winner of the prize, which is modeled on the Ansari XPrize, awarded in 2004 to give a leg up to cheap human spaceflight. The race to get to the moon, move around, and report back home is meant to foster cheap lunar access so that industry and government agencies can prospect for minerals or build resorts for space tourists. Along the way, though, science will benefit, says Andrew Barton, the prize's director of technical operations in Culver City, California. Movement over the surface and communication with Earth are basic technologies for many future science missions. Also, he notes, the competition offers two bonus

prizes that are at least partly scientific. The water discovery bonus (\$4 million) requires teams to unambiguously detect water on the surface and publish a peer-reviewed paper to prove it. "Water has been observed from orbit but no one has yet made a physical measurement on the surface," Barton says. For the Apollo Heritage Bonus Prize (\$4 million), teams must broadcast video and pictures from one of the Apollo landing sites, and Barton says data on how exposure on the moon has weathered the Apollo artifacts could have scientific value.

None of the potential finalists has declared an intention to look for water. That may be because they're most likely to find it in permanently shady craters at the poles, a difficult place for solar-powered rovers to reach. Nor is it easy to get a look at Apollo relics. Landing on the moon is imprecise, so the nearest Apollo site could lie far beyond the range of the teams' modest rovers. Closer landings risk damage to sites of historical importance, from the blast of a retro-rocket or a collision.

Part-Time Scientists, however, is planning to try. The team believes its rovers—developed with the help of the Audi car

One Lunar XPrize team wants to study the effect of space weather on the Apollo 17 rover.

company—have the necessary range. Karsten Becker, the team's chief technology officer for electronics, says they want to get up close to Apollo 17's lunar rover, which is made of materials including aluminum, fiberglass, nylon, and duct tape. "What's happened to that after 45 years in the space environment? Is it like new or in shreds from micrometeoroids?" he asks. Team Indus from India may go for a smaller, \$1 million bonus by visiting the site of an unmanned landing—China's Chang'e 3 lander and Yutu rover, which operated from 2013 to 2014.

Although the bonus prizes haven't generated a stampede to do science, most of the finalists have taken on one or several experiments. SpaceIL's magnetometer aims to help answer the question of where the moon's magnetic field comes from. Is it the relic of an ancient field, created by a churning iron core like Earth's, that was locked into its surface rocks when the core solidified? Or does it come from iron-rich asteroids that generate magnetic fields from the energy of their impact? As the Israeli craft orbits the moon and moves across the surface, the magnetometer will look for correlations between magnetic field changes and impact sites. "This mission may not settle the question once and for all, but we'll make progress," Aharonson says.

Another finalist, Team Indus, is holding an open competition for young people aged 25 and under to devise experiments that could point a way to sustainable settlements on the moon. The team was overwhelmed by 3000 entries from all over the world, including plant and microbial growth experiments, proposals to build lunar structures and radiation shields, and

even an attempt to brew beer on the moon. The team recently narrowed the field to a short list of 25, and those groups are now building prototypes that must be the size of a soda can and weigh less than 250 grams. In March 2017, up to eight experiments will be chosen to fly.

Moon Express is carrying a couple payloads: laser retroreflectors from a U.S.-Italian university group to precisely measure the Earth-moon distance for gravitational studies, and a 7-centimeter optical telescope for the International Lunar Observatory Association, a nonprofit aiming to show the power of observing in airless, ever-clear skies. The telescope will have open access for "citizen scientists."

Synergy Moon, an international team with offices in San Francisco, California, aims to blend the arts and sciences—perhaps with a holographic projector that will display artworks on the moon. The team claims it will study weathering of the lunar surface and the nature of the thin atmosphere above it using tiny autonomous robots they call lunar spiders and butterflies. These may be more artwork than instrument, according to a team blog post: "They will also be programmed for swarm behavior, to create random geometric and color patterns."

In general, it's best not to expect major payoffs for science, says Mike Ravine, a project manager at Malin Space Science Systems in San Diego, California, which builds instruments for NASA Mars missions. In 2000, Ravine attempted to get a private moonshot off the ground with Blast-Off! Corporation. The effort failed, but it taught him the challenge of trying to do science on a cut-rate mission. If the XPrize teams succeed, he says, "it would be great to wring some scientific value out of it. But it's a pretty high bar." ■

SCIENTIFIC PUBLISHING

In India, elite institutes in shady journals

Predatory publishers draw papers from faculty at top research centers

By Priyanka Pulla, in Bengaluru, India

India is home to a flourishing underworld of open-access journals that masquerade as legitimate scientific publications but publish papers with little or no peer review, while charging authors hefty fees. Many observers assumed that such bottom feeders were mostly attracting papers from institutions in academia's outer orbits. But a new analysis has found that many of the papers in predatory journals are coming from top-flight Indian research institutions.

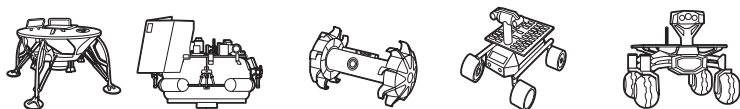
The finding has turned the spotlight on an academic culture in India that tends to prize quantity of publications over quality when evaluating researchers. This is an especially big problem in the life sciences, says K. VijayRaghavan, secretary of India's Department of Biotechnology (DBT) in New Delhi, which funded some of the research that ended up in predatory journals. "Biology, in general, has become ghastly, in that people are chasing the metrics," he says. "If you chase these surrogate markers of success instead of science, we have a problem."

Three years ago, a *Science* investigation traced the publishers and editors of scores of predatory journals to India (4 October 2013, p. 60). And last year, a team reported in *BMC Medicine* that of a selection of 262 authors published in predatory journals, 35% are Indian. Now, Gopalakrishnan Saroja Seethapathy, a graduate student in pharmaceutical chemistry at the University of Oslo, has looked at the authors' affiliations.

He and his colleagues randomly chose 3300 papers by Indian first authors from 350 journals flagged as predatory by Jeffrey Beall, a library scientist at the University of Colorado in Denver. In an analysis in the 9 December issue of *Current Science*, they report that more than half the papers were by authors from government-run and private colleges, hotbeds of me-

Moonward bound?

Five teams say they have booked launches to compete for the \$20 million Google Lunar XPrize, but they have different approaches to roving on the moon and doing science.



	SpaceIL	Moon Express	Synergy Moon	Team Indus	Part-Time Scientists
LOCATION	Israel	United States	United States	India	Germany
LAUNCHER	SpaceX Falcon 9	Rocket Lab Electron	Interorbital Systems Neptune	India's Polar Satellite Launch Vehicle	Likely SpaceX Falcon 9 (booking not yet confirmed by XPrize)
LANDER/ROVER APPROACH	Lander "hops" across surface with thrusters	Lander "hops" across surface with thrusters	Lander releases 30-centimeter-long, two-wheeled rover	Lander releases 5-kilogram, four-wheel-drive rover	Lander releases two 30-kilogram, four-wheel-drive rovers
SCIENTIFIC PAYLOAD	Magnetometer	7-centimeter telescope	Swarms of robotic "butterflies" and "spiders"	Up to eight 250-gram crowdsourced experiments	Multispectral camera

(GRAPHIC) G. GRULLÓN/SCIENCE

diocre research. But about 11% of papers were from India's premier research bodies, including institutions belonging to the Indian Council of Agricultural Research (ICAR), the Council for Scientific and Industrial Research, and the Indian Institutes of Technology. "Funding agencies have to be careful about where papers are published," says Subhash Chandra Lakhotia, a cytogeneticist at Banaras Hindu University in Varanasi, India.

Some blame the problem, paradoxically, on recent attempts to improve Indian research output. India's University Grants Commission (UGC), a body charged with setting educational standards, in 2010 made it mandatory for all faculty in higher education institutions to publish papers in order to be evaluated favorably. Pushkar, the director of the International Centre Goa in India, who goes by one name, says this move pushed teaching faculty with no expertise in research toward predatory journals, which provide an easy route to publication. "The research component in the performance metrics for faculty in teaching-focused organizations," he says, "is the reason why predatory journals attract so many submissions." In response to concerns, UGC announced that it would change its performance metrics and compile a list of peer-reviewed journals in which researchers would need to publish.

That's not the best solution, Vijay-Raghavan argues. "The fundamental problem is an ecosystem that values where you publish and how many papers you publish rather than what you publish. That needs to be changed," he says. Toward that end, DBT in 2014 required all published papers to be uploaded to a central repository so that they can be judged according to merit. The department also plans to launch a preprint repository, like the physics preprint site arXiv, to encourage sharing of findings before publication. Evaluating research by reading publications rather than focusing on numbers "will pull the carpet from under the feet of predatory publishers," VijayRaghavan says.

Some scientists feel that the predatory publishing scourge is overblown. ICAR Director General Trilochan Mohapatra in New Delhi argues that many publications classified as predatory could merely be little-known journals that charge publication fees. "There are many flaws with the *Current Science* paper," he says. "We will internally analyze this issue, see if a real problem exists at ICAR, and come out with our own study." ■

Priyanka Pulla is a writer in Bengaluru, India.

BIOCHEMISTRY

New blood tests make strides in detecting prion disease

Long after the European mad cow outbreak, researchers hope to screen blood supplies for misfolded proteins

By Kelly Servick

The "mad cow disease" epidemic that killed more than 200 people in Europe peaked more than a decade ago, but the threat it poses is still real. Eating meat contaminated with bovine spongiform encephalopathy and its hallmark misshapen proteins, called prions, can cause a fatal and untreatable brain disorder, variant Creutzfeldt-Jakob disease (vCJD). Thousands of Europeans are thought to be asymptomatic carriers, and they can spread prions through blood donations. So for years, researchers have sought a test to safeguard blood supplies.

This week, two teams bring that goal closer. They describe methods for detecting prions in blood that proved highly accurate in small numbers of samples from infected people and controls. "There is new technology to go forward, and it looks promising," says Jonathan Wadsworth, a biochemist who studies prion disease at University College London. "These are definitely very welcome papers."

Analyses of discarded appendix and tonsil samples suggest that as many as one in 2000 people in the United Kingdom carries abnormal prions—misfolded variations of a naturally abundant protein, which prompt surrounding healthy proteins to fold and clump abnormally. No one knows how many of these carriers will ever develop vCJD; incubation periods as long as 50 years have been reported. Once symptoms occur—first depression and hallucinations, and eventually dementia and loss of motor control—patients survive about a year. Four people are known to have contracted vCJD through a blood transfusion from an infected donor.

But screening for prions is difficult, be-

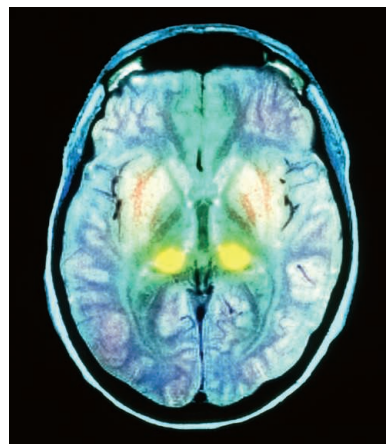
cause they are scarce in blood. Both new tests, described online in *Science Translational Medicine*, rely on a method that amplifies prions by culturing them with normal proteins and then agitating them with sound waves. That process breaks off fresh clumps of malformed proteins, which can more efficiently convert their neighbors. "Each can act as a seed to make this reaction go exponentially faster," explains Claudio Soto, a neurobiologist at the McGovern Medical School at the University of Texas Health Science Center in Houston, whose lab developed the technique.

In their new paper, Soto and his team tested their approach on blood from

14 vCJD patients and 137 controls. The test was positive in all vCJD patients and none of the controls. The second study, led by microbiologist Daisy Bougard at the University of Montpellier in France, used the same amplification technique, but first concentrated prions from the sample by capturing them with magnetic nanobeads. The technique flagged 18 vCJD patients out of 256 samples. It also detected prions in blood donated by two

vCJD patients before they showed symptoms—a first. "That's very encouraging," Wadsworth says.

But neither study analyzed enough samples to predict false positive rates across millions of samples in a blood bank, Wadsworth notes. "We have this ethical dilemma—how do you tell people they may have a fatal disease when we don't know whether the test is specific?" And because the United Kingdom accepts more than 2 million blood donations a year, he says, even a low false positive rate could needlessly deplete the donor pool. Both teams are now working to validate their tests on larger sets of samples. ■



Infected with prions, the brain of a vCJD victim reveals spongy areas (yellow).



ATMOSPHERIC CHEMISTRY

Scientists flag new causes for surge in methane levels

Swelling tropical wetlands and a decline in a natural atmospheric detergent are leading suspects

By **Paul Voosen**, in San Francisco, California

Carbon dioxide (CO_2) is not the only greenhouse gas on the rise. Since 2007, methane—which molecule-for-molecule has 30 times the warming effect of CO_2 —has risen by more than 3%. Befuddled scientists have tried to pin the growth on increased natural gas drilling, rising rice cultivation, and a surge in bovine belches. But none of these explanations has stuck.

Now, two more processes have gained ground as possible culprits, according to new work presented here last week at a meeting of the American Geophysical Union (AGU). In one scenario, methane's rise may come in part from a drop in hydroxyl, a chemical that acts as an atmospheric detergent; in the other, the gas is emanating from tropical wetlands flooded by heavy rains in recent years. Because climate change is expected to increase tropical rainfall, this methane could be a new signal that “the tropics are changing fast,” says Euan Nisbet, a climate researcher at Royal Holloway, University of London—and a warning that methane may continue to rise as the world warms in a positive climate feedback. “Methane may be a tropical parallel to Arctic sea ice,” Nisbet says.

Methane is normally held in check by the hydroxyl radical ($\text{OH}\cdot$), which scrubs nearly all of it out of the atmosphere within a decade. Formed in the presence of sunlight by water vapor and pollutants like ozone and

nitrous oxide, hydroxyl is hard to measure, because it persists for just a second in the air before it reacts away. Instead, scientists gauge $\text{OH}\cdot$ abundance by looking to proxies—chemicals that react with hydroxyl. One proxy is methyl chloroform, banned years ago for contributing to the ozone hole. A steady decline of that compound in the wake of regulations would suggest that hydroxyl is relatively constant, an assumption baked into most models of the methane rise. “ $\text{OH}\cdot$ tends to get ignored a bit in discussions even in the science community,” says Michael Newland, an atmospheric chemist who recently completed a postdoc at the University of East Anglia in Norwich, U.K.

But a close look at the methyl chloroform trend shows that the decline isn't as steady or certain as many have assumed. Instead it reveals bumps that could indicate a loss of $\text{OH}\cdot$, according to research presented at the AGU conference by Alexander Turner, a graduate student in atmospheric chemistry at Harvard University. The overall oxidative capacity of the atmosphere could be declining, allowing methane to linger longer, he says. Newland says that a fall in nitrous oxide from clean air regulations could have slowed the production of $\text{OH}\cdot$. But Turner cautions that the case for declining $\text{OH}\cdot$ is far from closed: With different assumptions, his sparse data could just as easily chart a rise in methane emissions rather than a hydroxyl decline.

And so scientists continue to look for un-

recognized new sources. Cows are unlikely, as their numbers saw their steepest increase between 2000 and 2006, when methane levels were flat. There's little evidence of thawing Arctic permafrost pumping out more methane. Expanded rice growing could play a role. But Ed Dlugokencky, an atmospheric chemist at the National Oceanic and Atmospheric Administration's Earth System Research Laboratory in Boulder, Colorado, sees another potential source: the heavy rains that washed over the tropics from 2008 to 2014, creating a surge in wetlands and methane-spewing microbes. “This is all very anecdotal,” says Dlugokencky, who gave a talk at the AGU meeting. “But I think it paints a consistent picture of what's going on.”

In recent years, researchers have noticed another clue to the puzzle: The carbon atoms in atmospheric methane molecules have shifted toward lighter isotopes. Because life prefers lighter carbon, the isotopes suggest to some scientists that the atmospheric rise must be due to extra microbial production, and not a boost due to leaked gas from fracking operations, which has a heavier isotopic signature.

But the lighter carbon could also signal that hydroxyl levels are falling, says Joe McNorton, a climate scientist at the University of Leeds in the United Kingdom. Because $\text{OH}\cdot$ prefers to react with lighter carbon, having less of it around would allow more of the light, microbial methane to linger in the atmosphere.

It's clear that methane scientists will need to come together to resolve this debate, as several factors are likely playing a role, says Eric Kort, an atmospheric chemist at the University of Michigan in Ann Arbor. “Trying to use any one single source or sink, or single measurement technique, to define exactly what's happening is perhaps too simple.” The payoff will be a clearer sense of the future: whether the methane rise is a long-term trend, driven by climate change, or a blip that could reverse next year. ■



PUBLIC HEALTH

Critics assail paper claiming harm from cancer vaccine

Vaccination rates fall as antivax campaigns gather steam

By Dennis Normile

Alarmed by “pseudoscience” that may bring “devastating” health consequences, two groups of researchers have asked the journal *Scientific Reports* to retract a paper that they claim undermines confidence in the human papillomavirus (HPV) vaccine, given to girls to prevent cervical cancer.

The 11 November paper describes impaired mobility and brain damage in mice given an HPV vaccine. The mice received doses that were proportionally a thousand times greater than that given to people, along with a toxin that makes the blood-brain barrier leaky. That protocol, critics contend, does not mimic what happens in the human body.

“Basically, this is an utterly useless paper, a waste of precious animals,” David Gorski, a surgical oncologist at the Barbara Ann Karmanos Cancer Institute in Detroit, Michigan, wrote on his Orac blog at scienceblogs.com. In an email to *Science*, the paper’s corresponding author, Toshihiro Nakajima of Tokyo Medical University, defended the work, stating: “Our manuscript was formally published after an intensive scientific review done by reviewers and by the editorial board of *Scientific Reports*.”

The tussle is the latest salvo in a widening global battle over the HPV vaccine. Originally licensed in 2006, the vaccine is now approved for use in more than 120 countries. Studies show it is already starting to reduce HPV infections, which are blamed for 528,000 cervical cancer cases and 266,000 deaths each year, with the greatest burden in developing countries. (Boys are also now getting vaccinated, as HPV can cause genital warts and various cancers.) But in several countries, girls have complained of debilitating symptoms,

Last July, women claiming side effects from the cervical cancer vaccine sued Japan’s government.

reminiscent of chronic fatigue syndrome, after vaccination.

These claims have attracted media attention, spawned antivaccination campaigns, and cut vaccination rates. More than 90% of Danish girls born in 2000 received at least one vaccine dose, but that rate has dropped year by year. Ireland also saw a drop in vaccination rates in 2015 and 2016. The trend is “alarming,” says Heidi Larson, who heads the Vaccine Confidence Project at the London School of Hygiene & Tropical Medicine.

Japan is the prime battleground. As in many countries, the HPV vaccine got off to a promising start there. The first vaccine was licensed in 2009; in April 2013, the ministry added the vaccine to its recommended list and offered it for free. Uptake was robust. Sharon Hanley, a cancer epidemiologist at Hokkaido University in Sapporo, Japan, and colleagues reported in *The Lancet* in 2015 that roughly 70% of girls born between 1994 and 1998 completed the three-dose vaccination course.

In spring 2013, however, a number of media outlets in Japan reported on alleged side effects. These include difficulty walking, headache, fatigue, poor concentration, and pain. That June, the health ministry suspended its “proactive recommendation” for vaccination, pending an investigation.

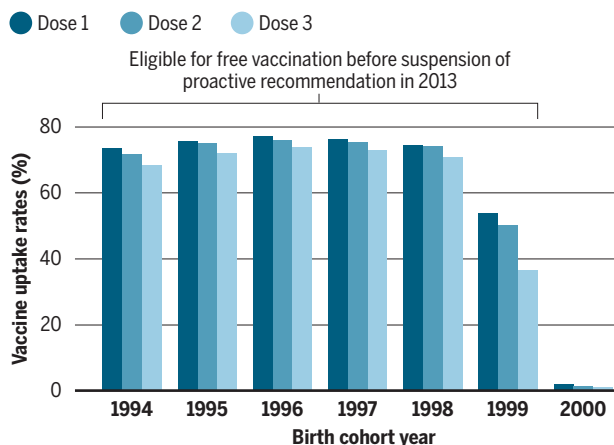
The following January a ministry panel concluded that there is no evidence for a causal association between the HPV vaccine and the reported adverse events. The European Medicines Agency and the U.S. Centers for Disease Control and Prevention have come to similar conclusions. Epidemiological studies indicate that the symptoms

reported by the vaccinated girls are found at equal rates in non-vaccinated populations. Yet Japan’s health ministry has never restored its proactive recommendation, which means that although the government pays for the shots, it has stopped urging local authorities to promote vaccination.

Vaccination rates have plummeted in Japan. Hanley says that in the city of Sapporo, the vaccination rate fell to just 0.6% of eligible girls, and she believes that nationwide, the rate is close to zero. (In a sign of growing trouble for vaccination, 63 women in July filed a class-action lawsuit against the government and vaccinemakers, seeking \$125,000 each in compensation for the alleged side effects.) Larson notes that health ministries

Off the cliff

After claims arose in 2013 that the HPV vaccine could cause debilitating side effects, the vaccination rate among women in Japan plummeted.



in other countries aggressively promoted vaccine safety after claims of side effects surfaced, keeping vaccination rates high. An official at Japan's health ministry says a decision on restoring the proactive recommendation is under review.

Nakajima's study is sure to inflame the debate. His group gave mice large doses of the HPV vaccine along with a pertussis toxin to help the vaccine slip into the central nervous system. The treatment, they found, impaired tail movement and locomotion. A postmortem revealed structural damage, increased cell death, and other abnormalities in the mice's brains.

Critics assail the study in a pair of letters to *Scientific Reports* and its publisher, the Nature Publishing Group. One, signed by 20 members of the HPV Prevention and Control Board at the University of Antwerp in Belgium, asserts: "This experimental set-up in no way mimics the immunization with HPV vaccines but is gross over dosage and manipulation of membrane permeability." A second letter, from David Hawkes, a virologist at the University of Melbourne in Australia, and two colleagues argues that the paper "lacks a clear methodology, adequate controls to control for bias, descriptions of results consistent with the data presented, or enough information for this study to be reproduced."

Nakajima defends his group's methodology, stating that they adopted a strategy similar to that commonly used in studying autoimmune encephalitis in mice. As for the dose, he wrote, "This is just the first paper and dose-dependency could be one of the interesting experiments in the future." He added that they are now preparing a detailed response to criticisms of their paper.

Vaccine proponents worry that the paper will embolden vaccine opponents. Nearly 200 tweets have mentioned it, with several mistakenly assuming it appeared in *Nature*. Both letters call on *Scientific Reports* to withdraw it. In an email to *Science*, a journal spokesperson confirmed having received the letters, and wrote, "We investigate every concern that is raised with us carefully and will take action where appropriate."

Even as opposition to the HPV vaccine gains momentum, evidence of its efficacy is accumulating. But with its paltry vaccination rate, Japan is unlikely to see any reduction in its current 9000-plus cases of cervical cancer and 3000 deaths each year. Worse, says Larson, Japan's suspension of the proactive recommendation "has been particularly applauded" by vaccine-critical groups in other countries. For women in Asian nations with weaker health infrastructure, Hanley adds, "The vaccine may be their only hope of prevention." ■

ENTOMOLOGY

Like birds, insects may travel in sync with the seasons

Long-term radar studies reveal masses of winged migrants

By Elizabeth Pennisi

Birds and human vacationers aren't the only creatures that take to the skies each year to migrate north or south. An analysis of a decade's worth of data from radars specifically designed to track airborne insects has revealed unseen hordes crossing parts of the southern United Kingdom—2 trillion to 5 trillion insects each year, amounting to several thousand tons of biomass, that may travel up to hundreds of kilometers a day.

The numbers, reported on p. 1584, are "stunning," says Silke Bauer, an ecologist at the Swiss Ornithological Institute in Sempach. "Wow," adds Larry Stevens, an evolutionary ecologist at the Museum of Northern Arizona in Flagstaff. "Can you image what these numbers look like in tropical settings, say, over the basins of the Amazon or the Congo?"

Although some insect migrations are well known (think monarchs), the new work takes a systematic approach to flying insects and hints that such mass movements are surprisingly common. These airborne invertebrates, their bodies packed full of nitrogen and phosphorus, could move significant amounts of key nutrients across the globe. "Insects are little creatures, but collectively they can have a big impact ... comparable in magnitude to large ocean migrations [of plankton]," says Lael Parrott, an environmental geographer at the University of British Columbia in Kelowna, Canada.

In the 1970s, U.K. entomologists began to use mobile radars to assess movements of locusts and other pests in developing countries. By the late 1990s, they had designed a permanent upward-facing radar system, based at Rothamsted Research in Harpenden, U.K., that automatically logs insects of different sizes. In one early discovery, Jason Chapman, now at the University of Exeter in the United Kingdom, and colleagues found that certain large butterflies and moths that dwell in northern Europe in the summer and in the Mediterranean in the winter take advantage of favorable winds to migrate (*Science*, 5 February 2010, p. 682).

Now, Gao Hu, from Nanjing Agricultural University in China, Chapman, and colleagues have surveyed data from 2000 to 2009 collected in Harpenden and two other U.K. radar sites. The radars recorded medium-sized insects (hoverflies, ladybird beetles, and water boatmen) and large ones (hawk moths, painted lady butterflies, and aquatic beetles) flying between 150 meters and 1200 meters high; balloon sampling flights helped provide estimated counts for smaller insects.

Among the medium and large insects, the radar documented 1320 mass migrations in the daytime and 898 at night over



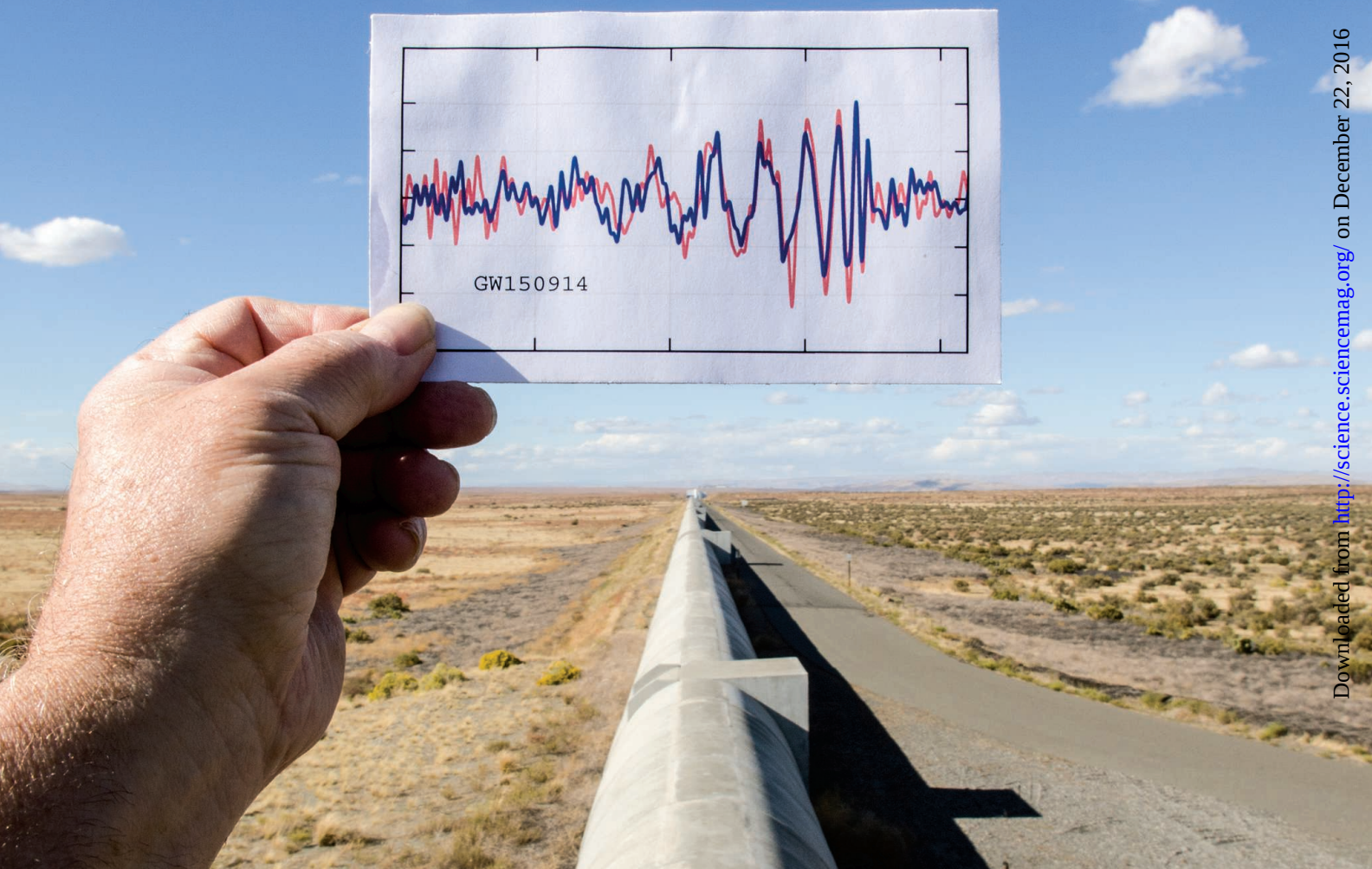
Hoverflies are one of many insect types that may migrate seasonally.

the course of the decade. These streams of insects, heading south in the fall and north in the spring, usually coincided with favorable winds, which swept them along at up to 58 kilometers an hour. That insects "have an idea of where they want to go to, when they want to go, and what winds are good [is] surprising for these tiny creatures," Bauer says.

It will take more data, from other sites, to convince some entomologists that many insects migrate seasonally like birds and mammals. A European initiative is tracking birds using weather radars, and its scientists hope to get the funding to monitor insects as well. Such studies could be critical, notes zoologist Eric Warrant of Lund University in Sweden. "If, due to human influence, a large fraction of the [insect] migrant population is wiped out, it might have catastrophic consequences for those particular ecosystems." ■

The cosmos aquiver

Detections of gravitational waves foreshadow a new way to eavesdrop on the most violent events in the universe *By Adrian Cho*



The discovery of ripples in space-time—gravitational waves—shook the scientific world this year. It fulfilled a prediction made 100 years ago by Albert Einstein and capped a 40-year quest to spot the infinitesimal ripples. But instead of the end of the story, scientists see the discovery as the birth of a new field: gravitational wave astronomy. In 1915, Einstein explained that grav-

ity arises because massive bodies warp space and time, or spacetime, causing free-falling objects to follow curved paths such as the arc of a thrown ball or the elliptical orbit of a planet around its sun.

Einstein then calculated that a barbell-shaped distribution of mass whirling end-to-end like a baton should radiate ripples in spacetime that zip along at light speed—gravitational waves. On 11 February, physicists working with the Laser Interferometer

Gravitational-Wave Observatory (LIGO)—twin instruments in Hanford, Washington, and Livingston, Louisiana—announced that they had seen just what Einstein predicted: a burst of waves created as two black holes spiraled into each other 1.3 billion light-years away.

The triumph was hard earned. Einstein himself vacillated for decades over whether gravitational waves should exist. Even if they did, the only source Einstein could imagine,

The carbon-copy signals seen by the LIGO detector in Washington state (shown) and its Louisiana twin.

two orbiting stars, would produce waves too feeble to detect. By the late 1960s, however, astrophysicists knew of much denser concentrations of mass. They had spotted neutron stars and dreamed up black holes, the ultra-intense gravitational fields left behind when massive stars collapse to nothing. Spiraling together, such things could, in theory, produce observable waves. In 1972, Rainer Weiss, a physicist at the Massachusetts Institute of Technology in Cambridge, set out a scheme to detect them with L-shaped optical instruments called interferometers, sowing the seed for LIGO.

Each LIGO interferometer has two 4-kilometer-long arms with mirrors at either end, housed in a giant vacuum chamber. By bouncing laser light between the mirrors, physicists can compare the arms' lengths to within 1/10,000 the width of a proton. A passing gravitational wave would generally stretch the arms by different amounts, and that's what the LIGO team spotted. The tight fit between that first signal and computer modeling validated Einstein's theory of gravity, known as general relativity, as never before.

Now, physicists are eagerly anticipating what may come next, because gravitational waves promise an entirely new way to peer into the cosmos. First, physicists hope to spot many more events. LIGO already has detected a second black hole merger and a third, weaker signal. The interferometers resumed taking data last month, and, if they can reach their design sensitivity, they may eventually see a black hole merger on average once a day.

Other instruments will soon join the hunt. The upgraded VIRGO detector in Italy should turn on early next year. Physicists in Japan are building a detector called the Kamioka Gravitational Wave Detector, and LIGO physicists plan to add a detector in India in the early 2020s. Three or more detectors together should be able to pinpoint a source in the sky by triangulation. That could help telescopes home in on the same event, and perhaps detect other signals from it. For example, if gravitational wave detectors sense the merger of two neutron stars and telescopes pick up light or x-rays from it, the signals together might offer clues about the exotic matter in neutron stars.

The detectors might even test wilder ideas about black holes. Quantum theory suggests a black hole might contain a hidden "fire-wall" that would obliterate anything that falls in. If so, merging black holes should produce gravitational wave echoes, some

theorists predict. Others speculate that a spinning black hole could generate a cloud of hypothetical particles called axions, which could generate gravitational waves by annihilating one another en masse.

Meanwhile, some astronomers are trying to detect gravitational waves in a different way. Within the hearts of large galaxies lurk supermassive black holes weighing hundreds of millions or billions of solar masses. When two such monsters merge, they radiate hugely powerful waves with wavelengths light-years long—thousands of times longer than instruments like LIGO can detect. To spot those waves, astronomers are turning to stellar timepieces called millisecond pulsars.

Pulsars—spinning neutron stars—emit incredibly regular pulses of radio waves. As long-wavelength gravitational waves buffet Earth, they should push the planet toward some pulsars and away from others. That motion would, in turn, shorten or stretch the time between pulses from pulsars in different directions, in an effect akin to the Doppler shift. The resulting variations and correlations among pulsars' timing should reveal the cacophony of long-wavelength gravitational waves, and the spectrum of longer and shorter waves would help physicists trace the rate at which galaxies formed and merged throughout cosmic history. Teams in the United States, Europe, and Australia hope to see a signal within 2 or 3 years—

although the U.S. effort is threatened by plans at the National Science Foundation to defund the two radio telescopes it uses.

Later, physicists hope to launch the Laser Interferometer Space Antenna (LISA). Three LISA spacecraft, cartwheeling in concert around the sun, would form a triangular interferometer with arms millions of kilometers long, enabling LISA to sense gravitational waves with wavelengths from millions to billions of kilometers—between LIGO's range of thousands of kilometers and the light-years of pulsar timing.

From those waves, LISA could track the mergers of smaller supermassive black holes with much greater precision than pulsar timing. It should be able to spot the long, slow windup of two black holes spiraling together before ground-based instruments like LIGO see the crashing finale. LISA could also detect stellar mass black holes falling into the supermassive black hole in the center of our Milky Way galaxy, enabling physicists to study that behemoth in great detail.

LISA was originally proposed decades ago as a joint mission between NASA and the European Space Agency (ESA), but the United States pulled out in 2011, citing budget con-

People's choice

Our readers weigh in, and surprise us with their top pick.

Visitors to *Science's* website were offered the chance to vote on a list of candidates for Breakthrough of the Year while *Science* editors and writers were finalizing their choices. A first round of voting narrowed the top candidates to five, and a second round, in which some 225,000 votes were cast, determined the final people's choice. In the end, a breakthrough in culture techniques that enabled researchers to keep human embryos developing in the lab for almost 2 weeks edged out *Science's* top choice, the detection of gravitational waves. We made human embryo culture an area to watch because we expect it will result in new findings about early human development and also spark debate on whether time limits on culturing human embryos—now 14 days in many regulations and guidelines—should be extended. Gravitational waves and human embryo culturing accounted for 75% of the final votes. The full results:

- 1 Human embryo culture **43%**
- 2 Gravitational waves **32%**
- 3 Portable DNA sequencers **13%**
- 4 Artificial intelligence beats Go champ **7%**
- 5 Worn-out cells and aging **5%**

straints. Now, NASA wants back in. ESA officials hope to launch the roughly \$1.5 billion mission in 2034—about the time physicists plan to build the next generation of ground-based detectors.

Mapping the sky with microwave telescopes, scientists might even spot, indirectly, traces of the longest and oldest gravitational waves, which rippled through the infant universe and now span the cosmos. Those primordial gravitational waves may have left an imprint on the afterglow of the big bang, the cosmic microwave background. Spotting them would help confirm that the newborn universe underwent an exponential growth spurt called inflation.

The discovery of gravitational waves has changed the scientific landscape. A new science beckons. ■

ON OUR WEBSITE

For more on the Breakthrough of the Year, including a video and a podcast, go to: <http://scim.ag/2016breakthru>.

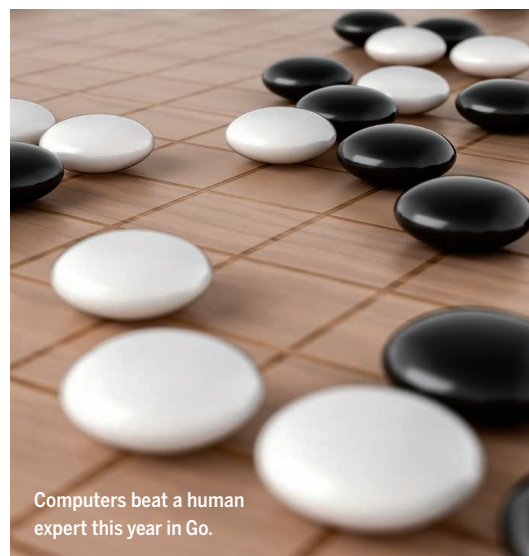
The exoplanet next door

Astronomers have found a small planet around the very closest star, Proxima Centauri. They are hailing the new world as our best opportunity to study in detail a planet outside our solar system, and are already straining to find out what it is like.

Tiny shifts in the frequency of Proxima Centauri's light revealed the planet, dubbed Proxima b. Astronomers monitoring the star detected a periodic increase and decrease in frequency every 11.2 days, caused by Doppler shifts in the light as the unseen planet repeatedly tugged the star toward and away from us. We don't yet know much about Proxima b except that it's at least 1.3 times the mass of Earth and orbits very close to its star—just 5% of the distance between Earth and the sun. That doesn't mean it's blisteringly hot. Because the star is a dim red dwarf, astronomers think the planet's surface may even be cool enough for liquid water to exist. But habitability is a long shot: Proxima Centauri is an unruly star that probably blasts the planet with a fierce solar wind, x-rays, and ultraviolet light.

Astronomers have been watching to see whether Proxima b passes in front of its star. If it does, the resulting dip in the star's brightness could reveal the planet's radius—which, combined with mass, gives its density—and starlight passing through its atmosphere could tell us what it's made of. But simple geometry makes such a "transit" unlikely—only a 1.5% chance—and searches so far have drawn a blank.

Scientists must now wait for better space- and ground-based telescopes planned for the next decade. But others are impatient: In April, the privately funded Breakthrough Starshot project announced plans to send a fleet of tiny spacecraft on a 20-year journey across the 40 trillion kilometers to the Alpha Centauri star system, which includes Proxima Centauri, and another private effort called Project Blue hopes to build a space telescope specifically to take pictures of planets in Alpha Centauri. —*Daniel Clery*



Computers beat a human expert this year in Go.

Artificial intelligence ups its game

This year, artificial intelligence (AI) passed a significant milestone when a computer program called AlphaGo beat the world's No. 2 Go player in a five-game match. It's not the first time that AI has surpassed human mastery of a game. After all, it was 20 years ago that IBM's Deep Blue first beat Garry Kasparov in a game of chess, toppling the world champion the following year in a six-game match. But that is where the similarity ends.

The rules of Go are more straightforward than those of chess: You simply place identical stones on a grid, capturing territory by surrounding

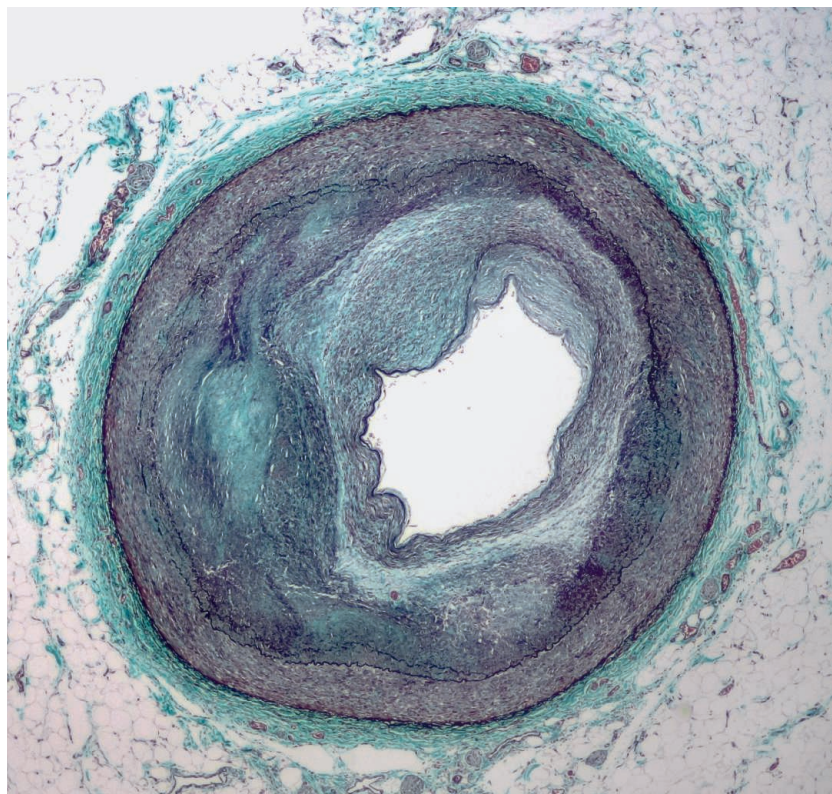
your opponent's positions. But that simplicity and openness result in an explosion in the number of possible moves for a player to consider—far more than there are atoms in the known universe. That makes it impossible for AI to beat Go masters with an approach like that used by Deep Blue, which relies on hand-coded strategies from chess experts to evaluate each possible move.

Instead, AlphaGo, designed by the London-based Google subsidiary DeepMind, studied hundreds of thousands of online Go games played between humans, using those sequences of moves as data for a machine-

An artist's concept of Proxima b orbiting the red dwarf Proxima Centauri.

learning algorithm. Then AlphaGo played against itself—or, rather, slightly different versions of itself—over and over, fine-tuning its strategies with a technique called deep reinforcement learning. The final result is AI that wins not just with brute-force calculation, but with something that looks strikingly like human intuition.

Most of the things we want AI to master involve a seemingly unmanageable number of possible decisions—walking a robot safely through a crowded room, routing driverless cars, making small talk with passengers. Because hard-coded rules fail for such tasks, AlphaGo's triumph shows just how powerful deep reinforcement learning can be. —John Bohannon



Removing worn-out cells might delay the buildup of artery-clogging plaques.

Killing old cells to stay young

Pricey plastic surgery won't stop you from getting old. Nor will dietary supplements, testosterone injections, or those wrinkle creams that imply they'll make you look 21 again. But this year, researchers demonstrated one way to postpone some ravages of time—at least in mice. When they selectively weeded out run-down cells, the animals lived longer and remained healthier as they aged.

The infirm cells the scientists targeted had undergone a partial shutdown known as senescence, in which they lose the ability to divide. Researchers think senescence may prevent worn-out, cancer-prone cells from initiating tumors, but it may also promote aging. As we grow older, more and more cells stop reproducing, potentially robbing our tissues of the ability to replace dead or injured cells. Senescent cells also discharge molecules that can cause problems such as abnormal cell growth and inflammation.

The first study showing that eliminating senescent cells can produce health and longevity benefits, at least in middle-aged mice, came out in February. Deterioration of the animals' hearts and kidneys slowed, and they didn't sprout tumors until later in their lives. Some age-related declines, such as in memory and muscle coordination, didn't abate. Nonetheless, the rodents outlived their contemporaries by more than 20%.

In October, the same research team took aim at senescent cells from the immune system that amass in artery-clogging plaques and may drive their formation. Removing these cells from mice that are prone to atherosclerosis reduced the amount of fatty buildup in the animals' arteries by 60%, even though the rodents gorged on fat-laden food.

The multibillion-dollar question: Will taking out senescent cells help humans stay young longer? Both studies used genetically modified mice that clear away their senescent cells in response to a particular compound—a technique that isn't feasible in humans. But researchers have created several so-called senolytic drugs that slay senescent cells without genetic tinkering. Next year, scientists will launch the first clinical trial of one of those drugs in people who have arthritis. —Mitch Leslie



Chimpanzees, bonobos, and orangutans have a skill thought to be uniquely human.

Mind-reading great apes

Great apes demonstrated a mind-reading skill this year that only humans were thought to fully possess. Known as theory of mind, it is the ability to discern desires, intentions, and knowledge in others. Some tests had shown that our close relatives have enough insight to, for example, deceive a fellow ape or recognize another's motives. But until now, they had always failed in tasks that require the ability to determine when others hold a false belief.

In the classic false belief experiment, a child watches someone hide a chocolate bar in a box and leave the room. Then someone else sneaks in and hides the candy elsewhere. Where will the first person look for it? Children who guess “in the original box” pass the test: Through what amounts to mind reading, they realize the first person holds a false belief. This skill is thought to be essential to deceiving, empathizing, teaching, and

perhaps even to using language.

This year, researchers conducted a version of the test on chimpanzees, bonobos, and orangutans. The apes watched a film showing a King Kong-like figure steal a rock from a man and hide it in one of two boxes. The man witnesses this action, but runs away when the Kong figure threatens him. While he is gone, Kong leaves with the rock. When the man returns, where will he search for it?

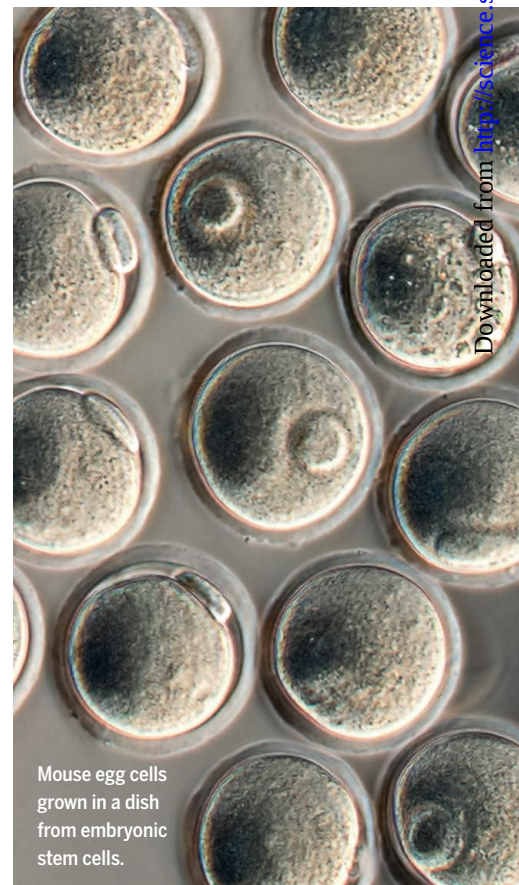
The researchers used infrared eye-tracking technology to see where the apes focused their attention. Almost all looked at the box where the man erroneously believes that his rock is hidden. Not everyone is yet convinced, but follow-up studies are likely to be in the works—and not just on great apes. The eye-tracking method can be adapted to other animals' faces.

—Virginia Morell

Proteins by design

Proteins are life's workhorses. They speed up vital chemical reactions, enable muscles to tug, carry out communication between and within cells, and defend against invaders. Given proteins' talents, researchers have long wanted to create their own versions. They have modified many existing proteins by making small tweaks to an organism's DNA code, but this year, they took protein modification to a whole new level: They created a suite of designer proteins unlike anything found in nature, setting the stage for novel medicines and materials.

Designing new proteins from scratch has been a hit-or-miss activity. It's easy enough to write any desired DNA code, but researchers have had no way of knowing how the novel strings of amino acids encoded by this DNA would fold into complex 3D shapes. That's a problem, because for proteins, shape dictates function. Recently, however, computational biologists have made heady progress in designing computer programs that



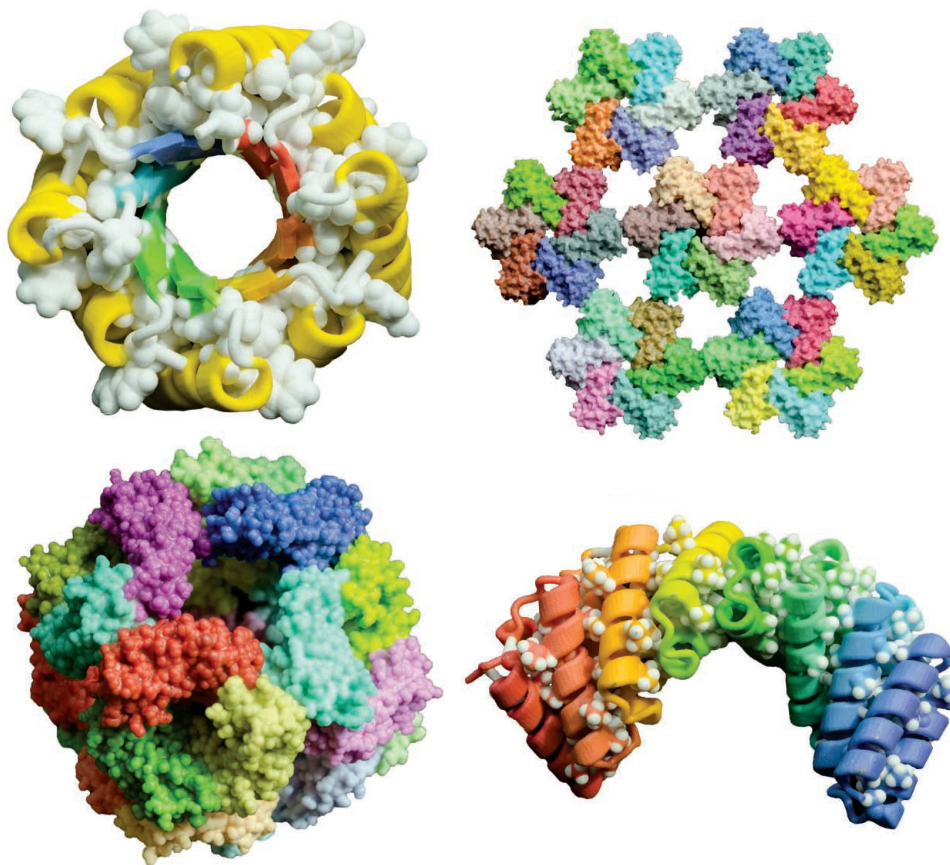
Mouse egg cells grown in a dish from embryonic stem cells.

accurately predict how designer proteins will fold. Those advances made possible this year's surge in designer proteins.

In February, a team led by researchers in Washington state used one such program to design what may become a universal flu vaccine, able to spark immune defenses to all flu strains simultaneously. In July, a team that included many of the same researchers created proteins that self-assemble into hollow cages, which someday could be filled with drugs or snippets of DNA to treat a range of diseases. Another team used a similar program to produce 3D, folded RNA molecules, which present a folding problem similar to proteins, as well as RNA-protein complexes, opening up new research possibilities.

Now, researchers want to use their skills to create everything from novel biosensors to new ways to remove carbon dioxide from the atmosphere. And because life makes only a tiny fraction of all possible proteins, protein designers have a vast new territory to explore. —Robert F. Service

Novel proteins, built using computer programs to predict how their amino acid strings will fold, are unlike anything produced in nature.

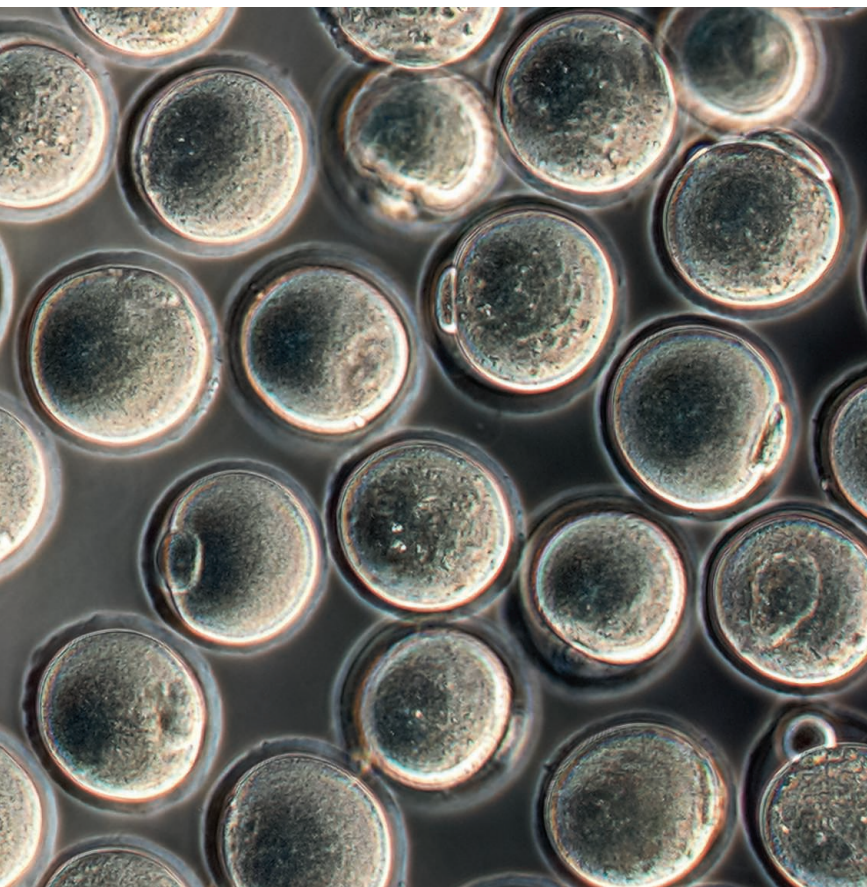


Mouse eggs made in the lab

Giving new meaning to the term “test-tube babies,” this year, researchers in Japan produced mouse pups from egg cells grown entirely in a lab dish. This long-sought achievement offers researchers a new way to study egg development and raises the more distant prospect of making human eggs in the lab from almost any type of cell, including genetically altered ones. That possibility has sparked hope for new infertility treatments, but it has also revived fears about designer babies.

In 2012, the same researchers took the first key step: They made fertile egg cells from stem cells. However, that method still required that the immature eggs be implanted back into a living mouse to complete their development. This year, researchers found a way to produce eggs entirely in the lab. Instead of implanting the immature eggs into a mouse, they cultured them inside clusters of cells taken from fetal mouse ovaries. The team then mixed the lab-grown eggs with mouse sperm and implanted the resulting embryos into foster mothers. Only 3% grew into full-term pups, but those pups grew into fertile, apparently healthy adults.

If scientists could perform a similar feat with human stem cells, it could lead to new options for some cases of female infertility. It may even be possible to turn stem cells derived from a man into eggs. Such possibilities are a long way from reality, but the ability to study egg development as it unfolds in a dish could produce insights that will have an impact in the clinic long before any human baby is born from a lab-grown egg. —Gretchen Vogel





Genomes from 83 Australian Aborigines showed that Australia was initially settled only once, not twice, as some had suggested.

A single wave of migration from Africa peopled the globe

The story of our species is driven by wanderlust. Born in Africa, *Homo sapiens* expanded into the far corners of the globe in the past 100,000 years, meeting and mingling with more archaic hominins already living there. But researchers have long debated how and when modern humans left Africa: Was it in a single migration or in repeated waves?

In 2016, a burst of genomic data all but clinched the case that most living people outside Africa descend from a single migration; any earlier migrations were mostly swamped by this last wave. In a trio of studies, researchers worked with aboriginal groups to collect and analyze hundreds of genomes from people living in far-flung corners of the world, including previously scarce samples from Australia, Papua New Guinea, and Africa. They tracked the ancient branching of populations recorded in the DNA.

One study analyzed 83 genomes from Australia, long considered a place apart. The DNA showed that, in contrast to previous sug-

gestions, Australia was initially settled only once. Moreover, the ancestors of Aborigines and Eurasians split from Africans around the same time, possibly about 70,000 years ago, suggesting a single exodus from Africa before the split. An independent study analyzing 300 genomes from 142 populations also reported a single wave out of Africa, which diverged into all living non-Africans starting perhaps 50,000 years ago, although the dates are imprecise.

The third study, which analyzed 379 genomes from 125 populations, reported mostly this same pattern, with a wrinkle: About 2% of the genome of Papua New Guineans may stem from a separate, earlier migration out of Africa, perhaps 100,000 years ago. Fossils show that some modern humans had made it to the Middle East by about this time, and a trail of stone tools in Arabia and India hints at such an early exodus. But the flood of new genomic data indicates that this earlier migration died out almost completely, leaving at most a trace in some living people. —*Elizabeth Culotta*

Genome sequencing in the hand and bush

Genome sequencing may be on the verge of becoming a ubiquitous tool in biology, both in the lab and—perhaps more important—in the field, thanks to a hand-held device that became widely available for the first time this year. It is already generating scores of research papers.

The device uses a breakthrough technology called nanopore sequencing to read the letters of DNA directly: As a strand of DNA passes through a narrow pore, the bases alter an ionic current in a unique, readable way. The big advantages

over traditional sequencing are that the startup cost for a nanopore sequencer is relatively low and it can, in theory, decipher unlimited lengths of DNA; the genome doesn't have to be chopped up and the sequences pieced together later by a computer. And because it's quick and portable—the device can churn out sequences in a matter of hours—it can potentially be used for biosurveillance, clinical diagnosis, and the investigation of disease outbreaks onsite.

Nanopore sequencing has been under development for years, and after more than a year of beta testing, a U.K.-based company, Oxford Nanopore Technologies, began marketing the first device this year. More than 30 papers based on the device

are already on the biology preprint site bioRxiv. Researchers have identified Ebola and other viruses in just a few hours, sequenced microbes in the gut, deciphered the 53-million-base genome of a fungal pest of maize, and, as they announced

3 earlier this month, sequenced a human genome. Even astronauts on the International Space Station used it to sequence a mixture of microbes in soil. Longtimers in the field point out that few of these advances have been documented in peer-reviewed publications, but others see this year as a turning point for a sequencing approach that is capturing the imagination of researchers who never considered genomic studies to be within their reach. —*Elizabeth Pennisi*

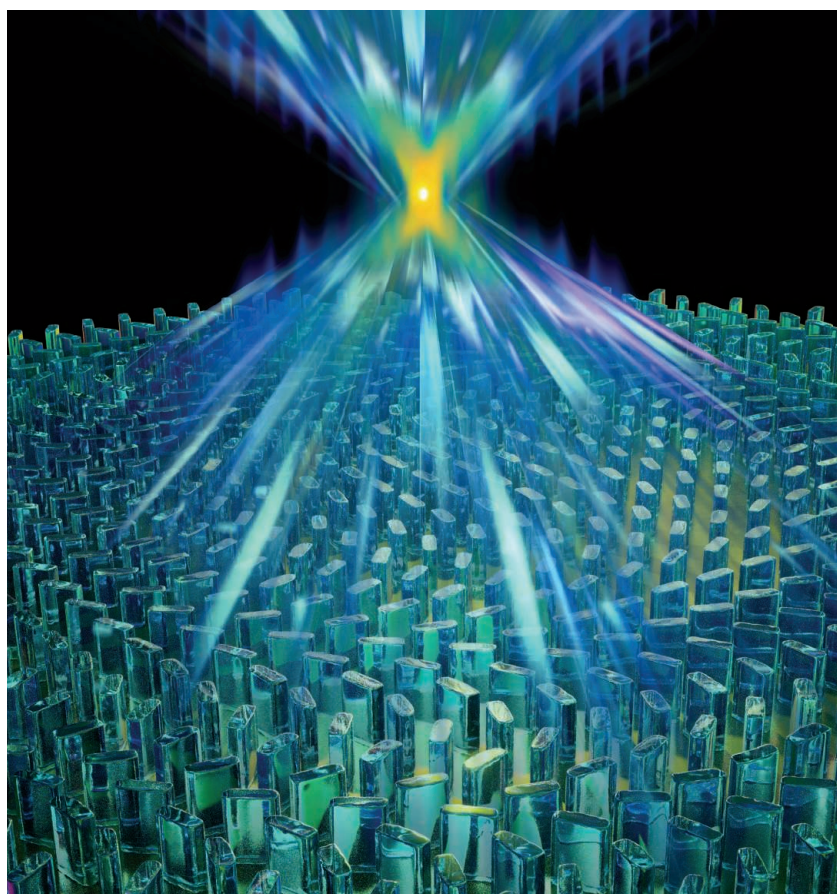
Metalenses, megapromise

Glass lenses were one of humanity's earliest high-tech innovations. They enabled Galileo to see Jupiter's moons, Antonie van Leeuwenhoek to spy microbes, and millions of people to just plain see clearly. But lenses are still made roughly the same way as they were centuries ago—by grinding and polishing glass and other transparent materials so that they focus light without aberration. Now, lens technology is poised to take a major leap. This year, researchers used computer chip-patterning techniques to create the first metamaterial lens, or metalens, that can focus the full spectrum of visible light. Because metalenses are cheap to produce, thinner than a sheet of paper, and far lighter than glass, they could revolutionize everything from microscopes to virtual reality displays to cameras—including the ones in your smartphone.

Metamaterials are composed of arrays of tiny pillars, rings, and other arrangements of materials, which work together to manipulate light waves as they pass by. In recent years, researchers have designed metamaterial-based “invisibility shields” that steer light around objects, as well as light filters and antennas. But previous efforts to make metalenses succeeded only with infrared and other long wavelengths of light; the patterning techniques didn't work as well with materials transparent to visible light.

This year, researchers figured out how to use a conventional chip-patterning technique, known as atomic layer deposition, to precisely pattern arrays of pillars of titanium dioxide. Just 600 nanometers high, the pillars are transparent to visible light, and they can focus it to produce a magnification of up to 170 times—as good as state-of-the-art-glass optics. The team tested its metalens techniques by using them to make holograms and carry out detailed spectroscopy, opening the way for other potential applications. Watch for the high-tech optics to make cellphones even sleeker, lead to new scientific instruments, and transform virtual reality headsets.

—Robert F. Service



Light passing upward through a metalens is focused by nanoscale fins.

Scorecard for 2016

Last year, *Science's* writers and editors picked three areas we considered ripe for new discoveries. How prescient were we? Our self-evaluations (the box scores) are mixed. Predictions for 2017 are on p. 1524.



WHO LET THE DOGS IN?

Last year, we predicted that 2016 might finally be the year we figure out where dogs came from. Scientists have fiercely debated whether our canine pals evolved from wolves in Europe or in Asia, and a new international collaboration of researchers promised to solve the mystery once and for all. We don't have an answer yet, but a study published this year suggests both sides of the debate might be right: Dogs may have been domesticated twice, once in Asia and once in Europe or the Near East.

FALLING BODIES

We recommended keeping an eye on a satellite called MicroSCOPE, which is testing whether two free-falling objects made of different materials accelerate at the same rate under Earth's gravity. Galileo supposedly tested that equivalence by dropping balls from the Leaning Tower of Pisa in Italy, and it is a cornerstone of Einstein's theory of gravity, general relativity. MicroSCOPE was launched successfully on 25 April, testing was completed this month, and researchers have begun an 18-month data run. Keep watching.

GRAVITATIONAL WAVES

It was a fairly safe bet a year ago when we made gravitational waves an area to watch for 2016. The Laser Interferometer Gravitational-Wave Observatory (LIGO) had recently undergone a major upgrade, physicists were sifting through early data, and rumors were swirling that they had detected ripples in spacetime: gravitational waves. Indeed, they had. LIGO has spotted two events so far, and many more are expected from LIGO and other planned detectors. These achievements earned our pick as *Science's* Breakthrough of the Year.

Areas to watch in 2017

Our recommendations for areas to keep an eye on next year range from the far reaches of the solar system to down-to-earth politics. Here are four topics we expect to generate a lot of interest in 2017.



President-elect Donald Trump's statements on scientific issues have alarmed scientists.

WHAT'S NEXT AFTER ELECTION SURPRISES?

For many scientists, the surprising election results on both sides of the Atlantic might rank as the 2016 Breakdown of the Year. In the United States, the election of a president who has dismissed the scientific consensus on climate change as a "hoax," said he would scrap dozens of science-based environmental regulations, and offered numerous fact-free observations on issues of the day does not augur well for the role of science in the Donald Trump administration. Researchers also worry because both Congress and the White House will be controlled by a Republican Party that has vowed to slash domestic spending and been hostile to research on some topics, including studies involving fetal tissue and embryonic stem cells.

In Europe, the Brexit vote for the United Kingdom to leave the European Union could reduce U.K. participation in European research projects and cut the flow of students, postdocs, and researchers to and from the United Kingdom.

Whether the worst fears will become reality is still unclear, however. Campaign rhetoric in the United States is rarely a blueprint for eventual policies. And in Europe, 2 years of negotiations will determine the conditions under which the United Kingdom will disengage from its European partners, providing some room for compromises.

Still, the year ended with worrisome signals for U.S. science. The nominee to head the Environmental Protection Agency has sued

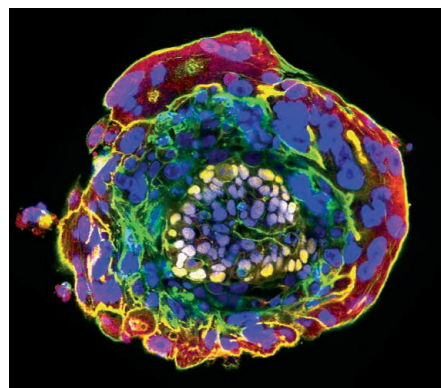
the agency to block regulations aimed at reducing greenhouse gas emissions, and the incoming Trump team demanded the names of Department of Energy employees and contractors involved in climate change research and policy. (Transition officials later called the request "unauthorized.")

For those reasons, *Science* is making the impacts on research of the U.S. and Brexit votes areas to watch. Watch them we will.

BREAKING EMBRYO LIMITS

Scientists in 2016 showed that they could keep human embryos developing in a lab dish for nearly 2 weeks, well past the point at which they would usually implant in the wall of the uterus. The achievement is likely to spark new research and renewed ethical debate. For decades,

1



Eleven-day-old human embryo tagged with fluorescent markers.

the widely accepted ethical limit for studying embryos in culture has been 14 days, well before the first signs of a nervous system begin to develop. Until this year, the barrier was theoretical; no one had kept an embryo growing for more than about 7 days. Should the limit now be extended to allow scientists to study the phases of development between 2 and 4 weeks—the period when organs start to form? Two groups in the United Kingdom held workshops this month to explore the issue, and some biologists hope that will help prompt a broad public discussion.

TESTING TIME FOR ZIKA VACCINES

A vaccine against Zika virus, which can cause devastating brain problems when it infects babies in utero, may prove its worth next year. In monkeys, almost every candidate Zika vaccine tested so far has provided complete protection—even when the animals showed relatively modest immune responses to the injections. A half-dozen, small-scale human trials of at least three different Zika vaccines have begun. They will assess the vaccines' safety and ability to stimulate the immune system. If the vaccines pass muster, they could enter efficacy trials early next year. Two concerns could slow development, however. Antibodies to Zika virus theoretically could interact with the closely related dengue virus and make people more susceptible to a deadly form of that disease. And Zika has spread so rapidly through Latin America—likely to be the key testing ground for a vaccine—that many people there may be immune, masking any benefit.

SEARCHING FOR PLANET NINE

My Very Educated Mother Just Served Us Nachos. Schoolchildren were just coming around to their Pluto-less, eight-planet mnemonic when, in January, astronomers shook it up with evidence for a Neptune-sized giant in a distant, 15,000-year orbit. They had not seen this new planet directly. Instead, they inferred its existence from its gravitational effects, seen in the clustered orbits of a handful of icy objects beyond Pluto. Astronomers have since noticed two more signs of the giant's influence: an emerging population of cometlike objects that swoop in and out of the solar system perpendicular to its plane, and a curious tilt to the sun. Several groups are using large telescopes to stare at patches of the sky where Planet Nine is likely to be lurking, looking for a telltale shift in a pinprick of light.

An attempted coup in Turkey led to street protests and mass arrests and firings.



Breakdowns of the year

Our picks for scientific breakdowns in 2016 involve scientists caught in political crossfires and a technology that fell short of commercial hype.

CRACKDOWNS IN TURKEY

Academic freedom has been under assault in Turkey for years under the country's hardline president Recep Tayyip Erdoğan. But the situation became dire after a failed coup attempt on 15 July. The government cracked down by arresting or firing tens of thousands of public employees suspected of supporting the coup—including thousands of higher education administrators and faculty. In the months that followed, some 15,000 teachers lost their licenses, the deans of all public universities were pressured to resign, and graduate students and post-docs based at Turkish universities were required to return from research abroad and reapply for funding.

Many expected the crackdown to abate—most of the deans have been reappointed—but the pressure has not let up. In November the government ordered fresh firings. And with powers granted by an ongoing state of emergency, Erdoğan abolished the nominally democratic system that faculty members have long used to choose university rectors. At Boğaziçi University in Istanbul, for example, the faculty voted overwhelmingly for Gülay Barbarosoğlu, an industrial engineer. Erdoğan instead installed his own choice: Mehmed Özkan, a biomedical engineer and the brother of a member of parliament from the ruling party.

THERANOS UNRAVELS

By the start of this year, the hype surrounding blood-testing startup Theranos of Silicon Valley in California had already begun to dissipate. Once valued at \$9 billion, Theranos promised to transform diagnostic testing with its “Edison” device, purported to test for conditions from vitamin D deficiency to cancer with just a few drops of blood. But an October 2015 article in *The Wall Street Journal* had reported problems with Edison, and this year, federal investigations and sanctions all but dismantled the company.

A report by the Centers for Medicare and Medicaid Services (CMS) described “deficient practices” at Theranos’s Newark, California, facility, including flaws in hematology tests that caused “immediate jeopardy to patient health and safety.” In July, CMS revoked the lab’s license and barred CEO Elizabeth Holmes from owning or operating a lab for 2 years. Theranos has appealed but faces separate investigations by the U.S. Securities and Exchange Commission, the U.S. Attorney’s Office for the Northern District of California, and several investors. In October, it announced it would close its clinical labs and testing centers and lay off nearly half its employees. Theranos says it is developing a new blood-testing device, the miniLab, but it is now seen as a cautionary tale for secretive scientific startups and overhyped consumer diagnostics.

CONGRESS VERSUS SCIENTISTS

Trust between several embattled research communities and senior Republicans in the U.S. House of Representatives reached a new low after two committees issued subpoenas aimed at forcing scientists, universities, and nonprofit groups to turn over records, including personal emails, unpublished data, and bank balances. Several science organizations, including AAAS (publisher of *Science*), objected, arguing the moves amounted to political harassment intended to chill scientific discourse.

One set of subpoenas targeted researchers working with human fetal tissue obtained from abortion providers including Planned Parenthood. Republicans leading a special investigative committee claim some tissue may have been sold, which would violate federal rules, but have released no evidence backing that allegation. Federal judges blocked some of the subpoenas after Planned Parenthood and others argued the panel had overstepped its authority.

The House science committee, meanwhile, added to the string of subpoenas it has issued over the past few years demanding information from scientists and others involved in climate and environmental issues. Committee chair Lamar Smith (R-TX), a climate change doubter, says he is using his subpoena power—exercised some 25 times since 2013—“to protect the freedom of scientific inquiry and to ensure regulations are based on sound science.” Many of the targets have resisted turning over documents. And critics say Smith’s demands reveal a less high-minded agenda aimed at undermining environmental regulations.

INSIGHTS



POLICY FORUM

ENTREPRENEURIAL ECONOMICS

Expand innovation finance via crowdfunding

Crowdfunding attracts venture capital to new regions

By Olav Sorenson,¹ Valentina Assenova,¹
Guan-Cheng Li,² Jason Boada,²
Lee Fleming^{2,3}

Crowdfunding (CF) platforms, such as Kickstarter (KS), offer a means of funding innovation, connecting inventors and entrepreneurs with a multitude of supporters, who each provide a small fraction of the amount required to fund the project. Although considerable funding for innovation has historically come

from venture capitalists (VCs), the entrepreneurs funded by VCs often mirror the investors in terms of their educational, social, and professional characteristics and end up concentrated in a small number of regions (1–4). Policy-makers have thus hailed CF platforms, hoping that they will expand access to entrepreneurial finance, including among women and minority innovators, and that the innovations funded will create jobs and spur economic growth (5). But if particular regions, or certain sorts of individuals, routinely pro-

duce better ideas (6), and VC concentrates on them, then CF might simply compete with professional investors to fund the same ideas. We find, however, that CF has been funding innovators in a large number of places that have typically been excluded from VC, and has also been expanding the geographic reach of VC itself.

We compare data from 2009 to 2015 on KS campaigns and on VC investments [see supplementary materials (SM) for details on all data and analyses]. One of the dif-



difficulties in juxtaposing these two sources of funding comes from the fact that many people run KS campaigns for projects that have no real possibility of being backed by VC, such as the creation of artwork, music, and dance. VCs, similarly, invest in some kinds of companies, such as biotechnology and medical devices, that fall outside the scope of the KS platform. VCs can also typically invest larger amounts of money than a KS campaign could raise. To ensure that any differences in geographic distributions of their activities do not simply stem from compositional differences in the kinds of projects funded, we restricted VC

investments to the smallest ones made in the youngest companies (seed and early stages), as these investments most closely correspond to KS campaigns. We also used a word-based distance metric to match categories of KS projects to industry codes for VC investments, aggregating all projects in a KS category and firms in a VC industry to create sets of words for matching. This matching identified 55,005 KS projects in categories similar to the industries in which VCs invested and 17,493 VC investments in industries engaged in activities similar to those of KS categories (for example, projects in the KS category “games” had descriptions similar to companies in the VC industry code “computer software”). We used these two sets to count matched KS projects and VC investments at the county-year level.

We first mapped the regions of successful KS campaigns (those that raised at least the target amount they sought) and VC investments (see the map). Although the typical KS campaign involves a smaller amount of money, these campaigns cover a broader swath of the nation. Several places with the largest number of successful campaigns have not been magnets for VC investments, e.g., Chicago, Los Angeles, and Seattle. By contrast, VC investments have been highly concentrated. Just four counties, located in the Boston area and Silicon Valley, account for 50% of all matched VC investments in our data.

To adjust for differences in population and other factors that might produce more investments in all types of innovative activity in some places, we calculated the relative intensity of KS versus VC dollars in each region. KS allocates a much larger share of its resources than VC does to the interior of the country, away from coastal population centers and traditional technology hubs. Even in the Boston area and Silicon Valley, KS investments end up concentrated in different parts. KS in the Bay Area, for example, goes disproportionately to Marin and Napa counties, whereas San Francisco and the peninsula counties receive more VC.

To assess whether VC differed from successful KS campaigns in their degree of geographic concentration and to determine whether these patterns might reflect the same underlying distributions, we calculated locational Gini (LG) coefficients [ranging from 1 (implying concentration in a single location) to 0 (implying perfectly even distribution across regions)] (7). VC investments in 2015 exhibited a much higher level of concentration ($LG = 0.98 \pm 0.002$ SE), than successful KS campaigns ($LG = 0.92 \pm 0.010$) ($t = 5.88$; $P < 0.01$).

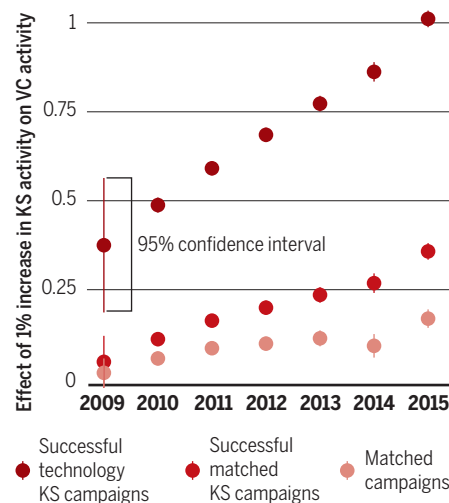
Although these maps and statistics suggest that KS expands access to financing outside

the traditional financial hubs of Boston, New York, and Silicon Valley, they only explore the cross section. Regions may vary on a number of dimensions that affect the prevalence of these funding sources. By focusing on the dynamics of investments within counties, we can hold these factors constant, to the extent that they do not vary greatly over time. Analysis of the sequential ordering of investments can also yield further insight into the relation between KS and VC.

Because KS campaigns involve much smaller amounts and can occur at earlier stages in the development of an idea, our temporal analysis focused on exploring the relation between KS campaigns and future VC

The Kickstarter effect

Effects of numbers of KS campaigns on the numbers of VC investments in a county, using KS campaigns in unmatched categories (such as art and music) to identify exogenous variation in KS activity.



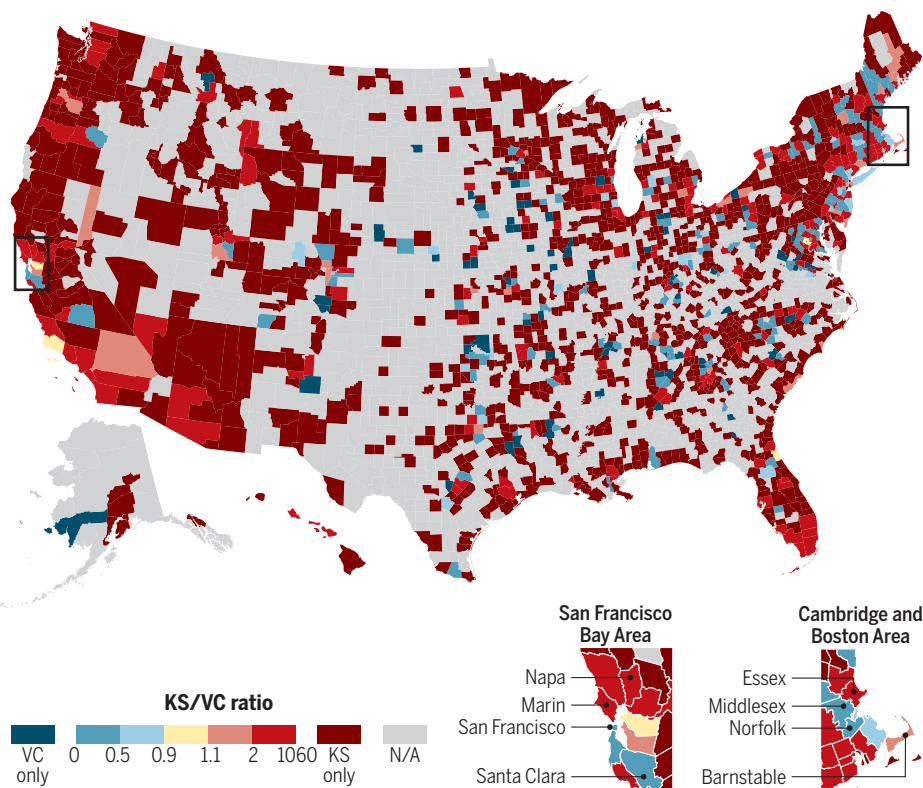
investments in a region. We first estimated the effects of successful KS campaigns on VC activity in a region in subsequent years. Our analyses focused on the numbers of successful KS campaigns and VC investments rather than dollar amounts, because these better reflect the number of innovations funded. Each of these models included controls for stable county-level characteristics, national-level factors at an annual level, and within-county changes from period to period in the number and quality of inventions.

A 1% increase in the annual number of KS campaigns in 1 year predicted a 0.097% increase in the annual number of VC campaigns in the following year, a 0.092% increase in the subsequent year, and about a 0.067% increase in the third year (all models $P < 0.01$) (see the graph). Successful campaigns may attract the attention of VCs to innovators in the

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Crowdfunding and venture capital at work

Distributions at county-level of matched Kickstarter (KS) campaigns, venture capital (VC) investments, and the ratio of the amount of KS to VC funding, 2009–2015. Increasing blue to red indicates a higher ratio of KS to VC funding.



region or to the specific people running these successful campaigns.

Although the temporal ordering of these estimates suggests that KS leads to future VC investment in the region, one might worry that some unobserved time-varying factors account for this effect. We therefore estimated the relation using instrumental variables (IVs) to try to eliminate the influence of endogenous or simultaneous confounding variables. Similar to a natural experiment, an instrument uses variation in the odds of being treated that appear random with respect to the outcome of interest. By predicting treatment on the basis of some third factor, unrelated to the outcome, and estimating the effects using these predicted values instead of the actual values, an IV isolates the effects associated with this exogenous source of treatment, which then has a causal interpretation, just as when one can assign the treatment experimentally.

To calculate our instruments, we used KS campaigns in categories distant from the industries in which VCs invest (i.e., the KS and VC categories had almost no common words in their descriptions). We used the number of KS campaigns in distant categories, such as art and dance, to predict the number of

KS campaigns in the categories that closely matched VC industries. We then used those predicted values—instead of the observed KS campaigns in matched categories—to estimate the effect of KS on VC.

We instrumented three different measures of KS activity using two different instruments: (i) the total number of KS campaigns (successful and unsuccessful) that closely matched VC, using the total number of KS campaigns that were distant from VC; (ii) the number of matched KS campaigns that were successful, and (iii) the number of successful matched KS campaigns in the technology category (five other nontechnology KS categories also matched to VC industries). Both (ii) and (iii) were instrumented using the number of successful distant KS campaigns. We estimated the effects for each year to explore whether the importance of KS in attracting future VC investments has been changing over time.

The results of these IV regressions are reported in the SM. Although the 2009 estimates for the overall number of campaigns and successful campaigns do not differ significantly from zero, by 2010, a 1% increase in the number of successful matched KS campaigns in 1 year predicted a more than 0.10%

increase in the number of VC investments in the same year. VC funding can quickly follow a successful KS campaign, as successful campaigns attract the attention of investors and as entrepreneurs tout their campaigns when pitching investors. A 1% increase in the number of successful KS campaigns in the technology category in 2009 predicted a 0.36% increase in the number of VC investments in the county that year.

Two notable patterns appear. First, the results become stronger as the KS measure becomes more closely restricted to projects of possible interest to VC investors. By comparing the predicted values using each of these models, one can estimate the proportion of the effect coming from each group of projects: Successful matched KS campaigns appear to account for all of the effect of the overall number (successful and unsuccessful) of matched KS projects, and projects in the technology category within the set of matched KS projects appear responsible for most of the effect (89.4%) of successful matched KS campaigns.

Second, these regressions suggest that the importance of successful KS to VC investments in a region has been rising over time. In 2015, for example, a 1% increase in successful KS campaigns corresponded to a more than 0.35% increase in VC investments in the same year; a 1% increase in successful KS campaigns in the technology category predicted a more than 1% increase in VC investments in the same year. As CF has gained legitimacy and entrepreneurs have learned how to use CF platforms, it may increasingly become a complementary source of funds to VC.

The results suggest an interesting and important relation between CF and VC. If these trends continue, the rise of CF may not only fund more innovation in a more diverse set of places but also expand access to VC and other forms of finance in these same regions. ■

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SUPPLEMENTARY MATERIALS

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CLIMATE CHANGE

Oceans on the edge of anoxia

Environmental crises can tip the ocean into O₂ depletion

By Andrew J. Watson

For the past several hundred million years, oxygen concentrations in Earth's atmosphere have been comparatively high (1, 2). Yet, the oceans seem never to have been far from anoxia (oxygen depletion) and have occasionally suffered major oceanic anoxic events (OAEs), recognized in the rock record through accumulations of dark, organic-rich shales (3). OAEs seem to be promoted by warm climates, and some have been associated with major environmental crises and global-scale disturbances in the carbon cycle. New insights into the causes of OAEs are now emerging (4, 5). Furthermore, ocean oxygen concentrations are declining in the modern ocean (6). A full-scale OAE would take thousands of years to develop, but some of today's processes are reminiscent of those thought to have promoted OAEs in the distant past.

Even today, the oceans are on the edge of anoxia: Every ocean basin has an oxygen minimum zone (OMZ) at a depth of a few hundred meters, where respiration consumes oxygen and depresses oxygen concentrations. Over large areas of the Eastern Pacific and the northern Indian Ocean, oxygen levels in the OMZ are close to zero. In modern times, some OMZs have expanded, a process called ocean deoxygenation (6). In addition, nutrient input from rivers has led to eutrophication and local anoxia in many coastal regions. An example of these "dead zones" is the Gulf of Mexico near the Mississippi Delta, but there are now several hundred anoxic coastal regions worldwide. Mostly, these are the result of human activities that have led to much-increased nutrient levels in rivers.

Why is the deep ocean deoxygenating? Human-caused eutrophication may be a con-

tributing factor, but climate change is also implicated. Warming promotes deoxygenation because it slows the formation of deep waters and decreases the solubility of oxygen in the surface. At a recent discussion meeting at the Royal Society in London, all these possible causes were discussed (7). Yet, none of them seems to fully explain the observed declines in oxygen, especially those seen in the tropical oceans.

As to the persistence of OMZs in the oceans past and present, a simple calculation, first made by Alfred Redfield (8), suggests

fixed by plankton from the atmosphere if it is in short supply.) Photosynthetic plankton then use up all the nutrients in the upwelling water, and the fixed organic matter sinks into the deep sea. There, it is respired back into inorganic carbon and nutrients by microbes, in the process consuming the oxygen supplied by the sinking water. Using the stoichiometry of carbon and nutrients now called the "Redfield ratios," Redfield found that the amount of oxygen consumed is almost equal to that carried down by the sinking water (8).

The oxygen demand in the interior of the modern ocean is thus constrained to be close to the oxygen supply. This near-equality seems paradoxical because demand and supply are set by two apparently independent variables: Demand is governed by the amount of phosphate in the deep ocean, whereas the supply is set by the amount of atmospheric oxygen that dissolves in surface water. A little more phosphate, and much more of the ocean would be hypoxic (low in oxygen). Doubling ocean phosphate would be sufficient to bring on a full-scale ocean anoxic event.

Past natural events are believed to have suddenly increased the supply of phosphorus, especially the massive outpourings of magma that form large igneous provinces (LIPs). Prominent OAEs occurred at the same time as, or very shortly after, the eruption of LIPs (4, 5, 9, 10). A possible mechanism is that the fast-weathering rocks employed by these events increase the supply of nutrients to the oceans for thousands of years, forcing the ocean into anoxia.

Once anoxia takes hold, it may be self-sustaining. Phosphorus is removed from the ocean through sedimentation, but if anoxic waters overlay these sediments they tend to leach out much of this phosphorus; in contrast, if the water is oxygenated the phosphorus stays in the sediments. Sediments in contact with anoxic water are thus an inefficient phosphorus sink and may even be a source of phosphorus to the ocean. Models suggest that once anoxia begins to spread over continental shelves

and slopes, this positive feedback may drive the ocean into prolonged deoxygenation that lasts hundreds of thousands of years (see the figure) (11, 12).

Since the industrial revolution, land-use changes, agricultural runoff, and sewage discharges have more than doubled the amount of phosphorus entering the ocean via rivers (13). The coastal dead zones that have developed as a result are often lethal to animal

Oxygen crises in the ocean

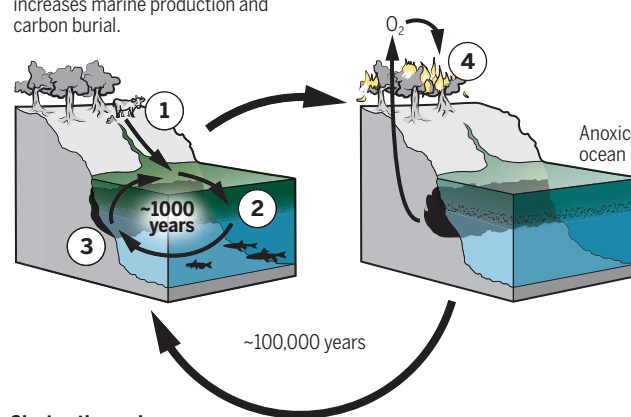
Major environmental crises can lead to oxygen depletion (anoxia) throughout the ocean's oxygen minimum zones, which become deadly for animal life.

Positive feedbacks

High phosphate inputs (1) enhance marine production, reducing oxygen in the OMZ (2). The resulting phosphorus release from sediments (3) further increases marine production and carbon burial.

Limiting processes

More burial of carbon leads to an increase in atmospheric oxygen, causing more wildfires that reduce forest vegetation (4). This lowers the phosphorus input to the oceans.



Closing the cycle

Over time periods of 100,000 years or more, the oceans recover from their deoxygenated state.

that the global ocean is chronically close to the edge of anoxia. When water leaves the sea surface, it carries oxygen absorbed from the atmosphere into the interior. An equivalent volume of deep water must upwell to the surface, carrying the limiting nutrient phosphate up from the deep ocean. (The deep waters must also carry up nitrate, but geochemists think of phosphate as the limiting nutrient because nitrogen can always be

life. However, the increase in nutrient input would need to be sustained for at least a thousand years to produce a change in phosphate levels sufficient to bring on a full-scale OAE. Whole-ocean anoxia is thus not an immediate global concern. If sustained for long enough, the deoxygenation occurring today could nevertheless have lasting negative consequences for the global environment.

On yet longer time scales, the paradox noted by Redfield may be explained by another feedback. A sustained increase in phosphate entering the ocean would increase not only anoxia but also marine productivity, causing more carbon to be buried in sediments. Carbon burial is the source of free oxygen because burial is the only process by which photosynthetically fixed carbon can escape reoxidation on a geologically short time scale. The resulting atmospheric oxygen increase would start to be significant on time scales of 100,000 years or more, eventually alleviating the ocean anoxia (see the figure). Rising molecular oxygen might also limit forest vegetation on land because of the increased prevalence of wildfire; given that forests increase the rates of weathering of continental rocks, limiting them would provide a negative feedback on the supply of phosphorus to the oceans, bringing the system back to a new steady state (14).

Atmospheric oxygen and ocean phosphorus are thus linked in a network of multiple feedback loops. Negative feedbacks help to explain the longevity and stability of atmosphere and ocean composition, but some feedbacks are of opposite sign and may at times destabilize the Earth system, as during OAEs. The role of these positive feedbacks in sustaining OAEs remains an open question, however, as does a complete description of the underlying causes of modern-day deoxygenation; conceivably, natural feedbacks may act to amplify the effects of global change on ocean oxygen concentrations. ■

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GLOBAL CHANGE

A stitch in time saves nine...billion

Acting now to limit global temperature change will help to ensure global food security

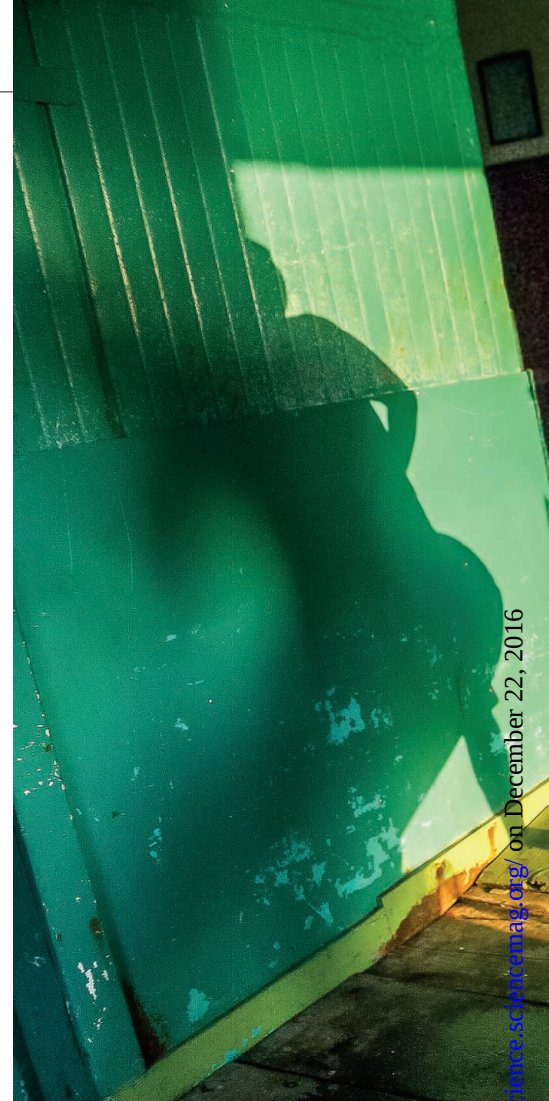
By Elizabeth A. Fulton^{1,2}

Human intuition often does not lead to thoughtful long-term decision-making (1). In partial remedy to this, a long history of storytelling conveys messages about what is worth remembering, worth doing, or best avoided. Stories also help to shape visions of what is possible and desirable (2). Scientific models are used in the same way. For example, the modeling study by Cheung *et al.* (3) on page 1591 of this issue indicates that making the effort to keep global temperature increases to 1.5°C would minimize impacts on marine food security and resilience. The work is an important addition to the discussion of the costs and benefits of keeping the average global temperature rise to less than 2°C.

A temperature change of 2°C does not seem like a lot, especially given much wider daily temperature ranges in many locations. However, in terms of global mean temperature, it marks a point where climate change would have broad-scale effects on many locations and aspects of life, ultimately leading to a less productive world with reduced capacity to support basic human needs (4). This was the motivation for the Paris Agreement, adopted by consensus on 12 December 2015 and ratified on 4 November 2016. The aims at the heart of this agreement highlight the basic tension in today's public policy decision-making: how to achieve economic development, meet basic human needs, and limit environmental impacts as the global population grows toward 9 billion by 2050. The agreement aims to hold the global average temperature rise to less than 2°C—and ideally less than 1.5°C—above preindustrial levels while also increasing adaptive capacity, resilience, and food security.

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Meeting these targets seems like a daunting task. The International Energy Agency has concluded that a \$16.5 trillion USD investment would be needed to meet the energy-related climate pledges of the agreement by 2030 (5). Nevertheless, models show the value of action and the costs of inaction. For example, Cheung *et al.*'s findings indicate that meeting the agreement's temperature target may also be the best means to deliver on food security and achieve resilient communities and economies.

The authors use models of changing species distributions, based on the physiological and habitat needs of each species and their capacity to move to suitable habitats as conditions change, to explore the potential future abundance and catch of 892 species currently fished around the world. They show that a global temperature rise of 1.5°C is enough to reduce fisheries catches on a global scale. As the temperature rise climbs from 1.5° to 3.5°C, global catch losses jump from 2.5 to 8.0%. Modeled changes in species diversity are even greater as species migrate or go locally extinct: Species turnover more than doubles from 8.3 to 21.6% as the temperature increase rises from 1.5° to 3.5°C.



A local fisherman takes his catch to market on the tropical Keys Island off the coast of Honduras. Tropical fish catches are likely to be much reduced as global temperatures rise.

The reductions in catch may not sound momentous, but as Cheung *et al.* show, the global total hides a lot of regional variation. Temperate and polar regions may see little change or even potential increases in fish abundance and catch, although these gains could be lost under more extreme climate change. In stark contrast, under a 3.5°C temperature increase, the authors forecast catch reductions of as much as 20 to 80% for tropical locations. The main reason for these large losses is the high turnover of exploited species in tropical locations, where many people subsist on seafood and depend on fishing for their livelihood (6). These findings are in line with other model results that have indicated much larger risks to coastal habitats, ocean processes, human health, and marine industries from rising temperatures and falling ocean pH (7). The model results thus underline how meeting the Paris target can help minimize both environmental and societal risks.

Research at a regional scale has come to similar conclusions for terrestrial ecosystem dynamics. In a recent study, Guiot and Cramer (8) combined models and reconstructions of past climate and ecosystem dynamics

to show that keeping to a 1.5°C temperature rise is the only means of staying within the range of conditions seen in the past ~10,000 years (the Holocene) around the Mediterranean. This is important because all human societies developed under Holocene conditions, which are assumed to mark our planet's safe operating space for humanity (9).

Modeling, as a tool for defining plausible options, has a history of more than 4000 years (10). Over the past decade, however, it has seen a renaissance, particularly in the diversity and scope of models and how they are used to inform decision-making. In the marine domain, trophic ecosystem models have been joined by qualitative, empirical, size-based, agent-based, and bespoke models of varying complexity to study fisheries management and biodiversity conservation in the context of global change (11, 12). The diversity of approaches helps to address scientific uncertainty and also provides modeling options even when the available data are poor. Marine ecosystem modeling thus not only provides global insights across fisheries (3) and other marine ecosystem services (7), but also helps to put fisheries and oceans management on a more sustainable footing (13).

Marine modeling, together with observed rapid changes in the world's oceans, has shown that both adverse change and beneficial human responses can occur with remarkable speed (7, 13). Constructive change typically has a cost and this often makes it unattractive, because our cognitive machinery amplifies the imagined disadvantages of change while underappreciating potential benefits (1). Nevertheless, at both regional and global scales, models are repeatedly showing us that overcoming or accepting short-term costs can lead to long-term gains in improved ecosystem status, food security, and societal resilience (3, 7, 8, 13, 14).

Current impact models (3, 8) largely focus on single drivers, such as temperature. Reliable methods of representing the complex drivers and effects of global change are yet to be developed. Nonetheless, the futures that the models present already provide an impetus to act. Current climate pledges under the Paris Agreement do not provide the reductions in greenhouse gas emissions needed to keep temperatures below 2°C, let alone 1.5°C (15). But for those people relying on the close to 25 billion seafood meals lost each year if we let temperatures rise by 3.5°C, the imperative to act seems clear. Humans today are better equipped to make evidence-based decisions than at any point in our collective history, and all that evidence tells us that deferring the decision to act is a costly course to take. ■

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QUANTUM OPTICS

Optical circulators reach the quantum level

The spin state of a single atom can route light signals in different directions

By William J. Munro^{1,2} and Kae Nemoto²

Optical circuits for information processing offer higher data rates (channel capacities) and lower power consumption as compared with those of electronic circuits.

Many optical devices (such as switches and isolators) have analogous electronic components (transistors and diodes), but some have special capabilities. For example, optical circulators form part of an unusual class of components known as nonreciprocal devices (1)—their optical properties depend on the direction of light passing through them. A three- or four-port optical circulator routes light entering from any port to exit only from the next. Bulk optical implementations rely typically on nonreciprocal polarization rotation via the Faraday effect, in which a magnetic field breaks symmetry (2). However, the drive to miniaturization that has led to nanophotonic integrated circuits and waveguides has not included optical circulators until now. On page 1577 of this issue, Scheucher *et al.* (3) demonstrate a fiber-integrated photonic circulator that can work even at the single-photon quantum level.

The fiber-integrated circulator used in this demonstration (see the figure) is formed from a single-spin-polarized atom (whose quantum state controls the operational direction of the circulator) coupled to the evanescent field of a whispering-gallery-mode microresonator (4), with two coupling fibers interfacing it. In order to understand how this device works, we can begin with the whispering-gallery-mode microresonator. The evanescent field that surrounds the resonator (a field that diminishes with distance from the resonator) exhibits a substantial polarization component along the light's propagation direction,

which oscillates 90° out of phase with respect to the transverse component. The resulting circularly polarized evanescent field exhibits a local photon spin that is orthogonal to the field's propagation direction and directly linked to it. If the field propagates in

depending on the spin state of the atom. In order to exploit this effect, Scheucher *et al.* interface the resonator with two tapered fiber couplers (see the figure). Depending on its propagation direction in the coupling fiber, the light then either couples into the resonator and exits the latter via the

other fiber, or it cannot enter the resonator and continues on its way. This process selects for the desired functionality of the optical circulator: Photons from port i ($i = 1, 2, 3, 4$) are routed to port $i + 1$ (2, 3, 4, 1) but not port $i - 1$ (or vice versa for the opposite spin state).

This whispering-gallery-mode fiber-integrated circulator is controlled by the quantum state of a single atom and can operate at the single-photon level, unlike many potential alternatives. Given state-of-the-art technology, this device can in principle be extremely low loss (0.3 dB) and have near-unity efficiency. These features make this router ideally suited for quantum information processing tasks including quantum communication (5), computation (6), and simulation and could be integrated into quantum photonic chips. Further, the principles underlying this nonreciprocal device could be used to construct optical diodes (isolators) (7) and fiber switches (8). The most likely and intriguing application would be fully functional quantum-entangling gates (9), both photon-photon and photon-atom if the atom is in a spin superposition state. This latter case is particularly interesting because it

gives excellent ways to bring nonlinearities to single-photon quantum technologies in an efficient and compact manner. ■

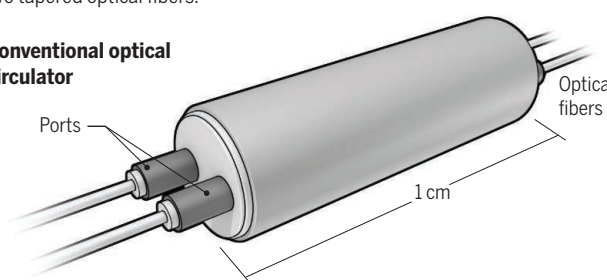
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Downsizing optical circulators

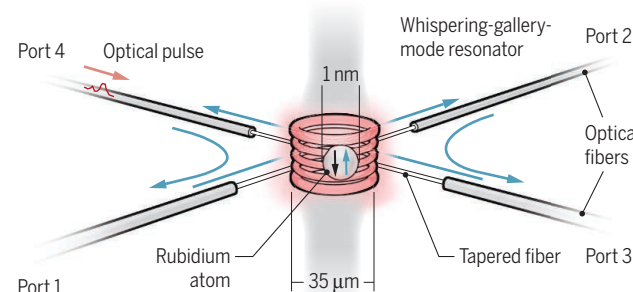
A large conventional optical circulator (top) is shown in contrast to a photonic four-port circulator (bottom), in which a rubidium atom is coupled to the whispering-gallery mode of a bottle microresonator with two tapered optical fibers.

Conventional optical circulator



Photonic optical circulator

The spin state of the atom determines the paths of pulses through the circulator. The paths shown are for spin up; all paths reverse direction for spin down.



the clockwise sense, the electric field vector rotates counterclockwise—it is σ^- polarized. If the field propagates in the reverse sense, the field vector rotates clockwise and is σ^+ polarized.

The spin-polarized atom acts as a polarization-dependent scatterer that has dramatically different interaction cross sections for the σ^- and σ^+ -polarized light fields. By introducing such a spin-polarized atom into the resonator, the resonator light field can be suppressed for one sense of circulation. At a given frequency, light can only circulate in the clockwise or counterclockwise sense,

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EVOLUTION AND DEVELOPMENT

The “tao” of integuments

Hair follicle and sweat gland fates can be switched by morphogens at specific skin regions or developmental stages

By **Yung Chih Lai** and **Cheng-Ming Chuong**

The integument forms the interface between an organism and its environment. It serves diverse functions such as communication, endothermy, defense, and flight. During vertebrate evolution, various integumentary organs, including hairs, feathers, glands, and teeth, have evolved to help animals adapt to evolving environmental changes (1) (see the first figure). These ectodermal organs form through epithelial-dermal interactions. Classic tissue recombination experiments have demonstrated that the dermis specifies the organ phenotypes within a developmental time window (2). Yet the underlying mechanisms remain unclear. On page 1551 of this issue, Lu *et al.* (3) reveal the specific molecular mechanism whereby an epithelial placode can be guided to form either a hair follicle for its architecture or a sweat gland for its secretory function.

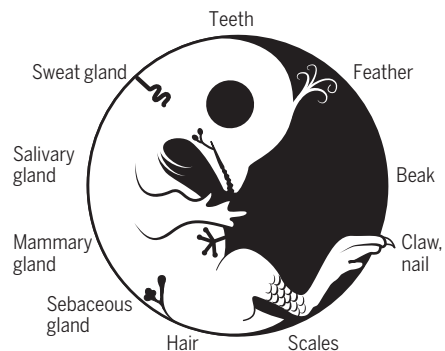
It is instructive to consider nature’s way of integumentary organ design—the “tao” of this process. Periodic patterning has evolved as an effective integument organization principle (4) (see the second figure).

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This is achieved by partitioning the integument into numerous elements (e.g., on average, >30,000 hair follicles in a mouse). Each element contains its own stem cells and undergoes cyclic regeneration, thus allowing it to regenerate after wear or injury. Further, each element can be independently controlled by modulating the stem cells to form different appendage types under different physiological or hormone conditions (e.g., rooster or hen feathers) and in different body regions (e.g., downy feathers become flight, tail, or contour feathers) (5). Collectively, this is an effective way to build complex yet adaptable integuments.

Evo-devo of integuments

A prototype animal with diverse forms of integumentary appendages.



For filter-feeding animals such as baleen whales, modification of the integument blocks tooth formation in the mouth cavity to permit hair bundles to form instead.

Stem cells enable a hair or feather follicle to undergo cyclic renewal, and each cycle is composed of growing and resting phases. The length of the hair filament is a function of the duration of the growth phase, which is regulated by fibroblast growth factor 5 (FGF5) (6). This mechanism enables closely related species to quickly adapt to cold or warm environments. Yet this is a quantitative change. What about qualitative changes that alter organ functions?

Lu *et al.* demonstrate how hair follicle and sweat gland fates can be switched. In mice, sweat glands are confined to the paw regions, and the dorsal skin can only form hair follicles. Transcriptome profiling suggested distinctly different amounts of several bone morphogenetic proteins (BMPs), WNT proteins, and FGF proteins in regions producing hair follicles versus those producing sweat glands. Previous experiments already showed that suppressing BMP signaling could convert sweat glands in mouse paws into hairs (7). Here, the authors used inducible and tissue-specific transgenic mouse and lentivirus technology to perturb morphogen signaling levels at different times and in different locations. Suppressing BMP signaling caused placodes in the ventral foot to switch fates to form hairs, whereas increasing BMP signaling guided placodes on the dorsal skin to switch fates toward sweat glands.

BMP works in a circuit with WNT, FGF, and sonic hedgehog (SHH), and the authors show that BMP signaling in the dermis differentially elevates WNT expression to higher levels in the ventral foot than in the dorsal skin. In WNT-responsive dermal cells, the expression of several FGFs are highly up-regulated in the ventral foot relative to the dorsal skin. Ectopic FGF18 increased expression of the glandular marker K18, decreased expression of the hair follicle marker K17, and caused hair loss in the dorsal skin. Furthermore, ectopic expression of FGF18, BMP4, and BMP5 in the dorsal epidermis induced *Engrailed-1* expression in the ventral foot but not in the dorsal epithelium, and caused a more glandular fate. SHH is highly expressed in the hair relative to sweat gland placodes. Increasing SHH signaling in the ventral foot epidermis induced or repressed the expression of hair-specific markers and gland-specific markers, respectively. Thus, Lu *et al.* identify a molecular circuit in the ventral foot that works synergistically to form sweat glands.

One of the critical events in the evolution of early humans is the expansion of

the sweat gland-producing region to cover the body (8). This alteration allowed people to have improved heat tolerance, which enabled them to run long distances in big game hunting while maintaining their body temperature. Does similar circuitry function in controlling hair versus sweat gland fate determination in humans? Lu *et al.* found high BMP activity spikes around 17 weeks of gestation, shifting placode fates from hairs to glands. Thus, similar sweat gland-forming circuitry is activated by spatial signals in mice but by temporal signals in humans. The next challenge will be to find out how BMPs are regulated spatiotemporally.

There are many more examples in which modification of the integument helps animals adapt to their changing environment. For example, the tooth changes shape in related rodents to enable optimal use of their diets (9). In filter feeding, baleen whales' tooth formation is shut off and modified hair bundles form in the mouth cavity instead (see the photo). At the macroevolutionary level, changes in integument organs help define a whole new vertebrate class (10). For example, the conversion of scaly integument to feathers in feathered dinosaurs was the prelude to the birth of the bird as a whole new class (11, 12). And in mammals, the evolution of mammary glands helps define the mammalian class (13, 14). Thus, the ensemble of modules (hair or feather follicles) allows the generation of a complex integument that is highly adaptable. Modulation of integument phenotypes can occur

at the genomic level with consequences at the evolutionary scale, or at the epigenetic level with metamorphic changes in the same individual, or in the same species but at different ages, sexes, and seasons (15).

Understanding the "tao" of the integument can also lead to medical benefits. With the progress of stem cell biology, scientists now have access to ectodermal progenitors. These new insights will help our efforts to guide these progenitors into new integument organ phenotypes for regenerative medicine. ■

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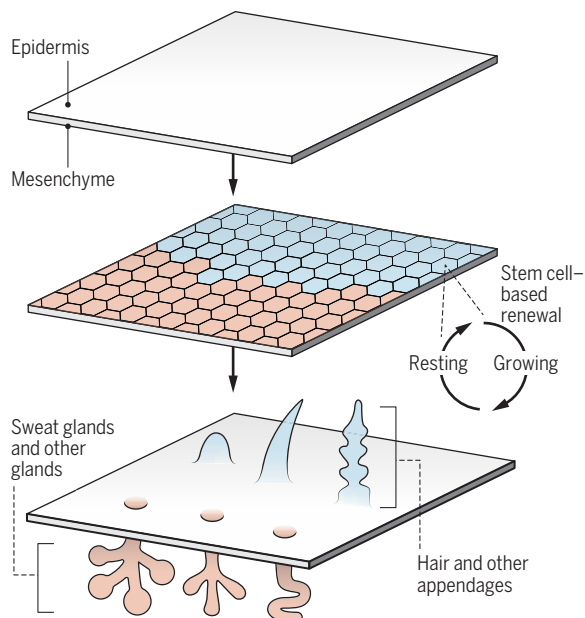
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Organizing to adapt

Periodic patterning enables the formation of complex yet adaptable integument organs.



Simple beginnings

In very early vertebrates, the integumentary surface was smooth, containing an epidermal and mesenchymal layer.

Multiple elements

Periodic patterning evolved to partition integuments into multiple elements, each with stem cells that allow cyclic renewal. Regional specificity also evolved (blue and red).

Integumentary appendages

Morphogen signaling at different developmental time points and locations evolved to modulate the form of integumentary elements, enabling them to adapt to changing environments.

GENOMIC MEDICINE

"Pheno"menal value for human health

Rare genetic variants are linked to electronic health record phenotypes at a population scale

By Daniel J. Rader and Scott M. Damrauer

One of the greatest challenges of the biomedical enterprise is to link human genetic variation with phenotypic traits at a population scale. Such efforts have myriad benefits, including the illumination of basic human biology, the early identification of preventable and treatable illnesses, and the identification and validation of new therapeutic targets, thus enabling the promise of precision medicine to improve human health. On page 1549 of this issue (1), Dewey *et al.* report findings from their effort to couple whole-exome sequencing (WES) with longitudinal electronic health record (EHR) phenotype data in more than 50,000 individuals (DiscovEHR). They identified hundreds of individuals with rare "putative loss-of-function" (pLoF) gene variants linked to novel phenotypes. On page 1550 of this issue, Abul-Husn *et al.* (2) report on a companion study that identified many participants with familial hypercholesterolemia who had not been diagnosed or treated. These results demonstrate the enormous potential of this approach for promoting scientific biomedical discovery and influencing the practice of clinical medicine.

Previous studies have used high-throughput identification (silicon chip-based genotyping) of known common variants in EHR cohorts (3) or applied WES [a technique for sequencing all the expressed genes (exome) in a genome] for rare variant discovery to case-control cohorts for complex diseases (4). However, the approach by Dewey *et al.* and Abul-Husn *et al.* is notable because of the application of large-scale WES to a phenotypically rich EHR cohort. Popu-

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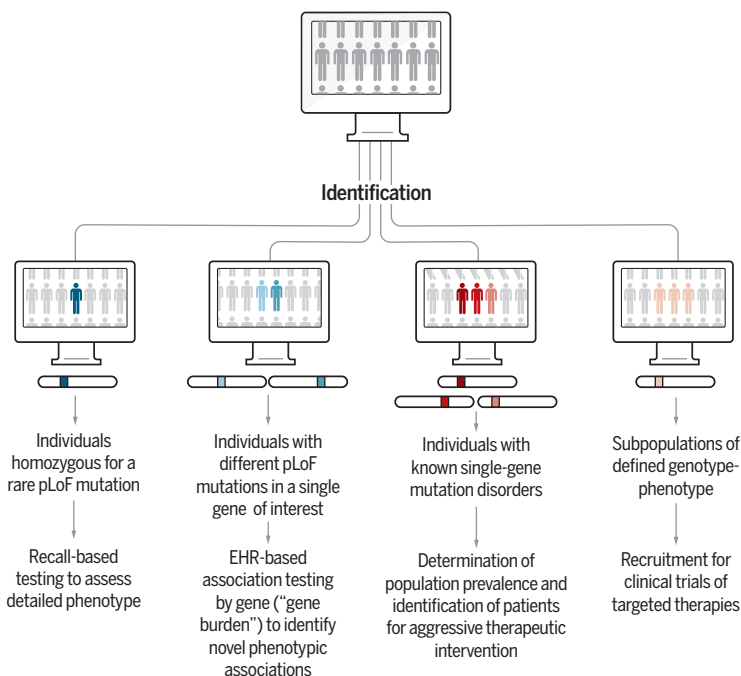
lation-scale deployment of WES has been facilitated by advances in next-generation sequencing and decreasing costs. Relative to chip-based genotyping, WES permits the detailed identification of rare and private (specific to an individual) protein-altering genetic variants that can cause Mendelian conditions (where a mutation in a single gene causes a disease) as well as contribute to complex multigenic traits and disease. By focusing on protein-coding sequences, WES simplifies the link between variant-trait association and mechanistic causality. This approach relies on algorithms for predicting the effect of genetic coding variation on protein function (5), but these techniques are imperfect. The increasing use of WES highlights the need for high-throughput technologies that can screen genetic protein-coding variants to provide experimental evidence of pathogenicity (6).

There are a number of unique opportunities derived from applying WES to EHR “biobank” cohorts (see the figure). WES allows the identification of rare pLoF homozygotes for whom EHR data can facilitate a deep dive into the associated phenotypes. An example, shown by Dewey *et al.*, is an individual who is homozygous for a pLoF in the *CSF2RB* gene, which encodes colony-stimulating factor 2 receptor beta common subunit. Defects in *CSF2RB* are associated with pulmonary alveolar proteinosis, and this individual displayed a phenotype consistent with this condition. There is also the potential for recall-based deep phenotyping, in which persons with rare pLoF genotypes can be asked to participate in additional targeted experiments. It is important to note that participants in DiscovEHR agreed to be recontacted for additional phenotyping—an approach that should be considered standard for large biobanking efforts of this kind.

The breadth of EHR phenotype data readily permits the application of phenotype-wide association studies (PheWAS), in which specific genetic variants can be interrogated in an unbiased manner for their association with a variety of phenotypes (7). Although PheWAS was initially developed for common variants of sufficient frequency in the population to permit the identification of associated traits, WES

Mining rare variant phenotypes

Linking dense EHR-derived phenotypes with whole-exome sequencing data can address a broad range of biomedical questions and goals. Through a “genetics first” approach, individuals who carry rare genetic variants of interest can be rapidly identified for subsequent EHR- or recall-based investigations, clinical trials, and preventive interventions.



permits its extension to rare variants of potentially greater phenotypic effect by “gene burden” testing. This method collapses pLoF variants at the gene level for statistical testing to uncover novel phenotypes associated with genetically impaired protein function. Dewey *et al.* identified a number of genes in which a “burden” of pLoFs was significantly associated with one or more EHR traits. Most of these traits were quantitative laboratory values (such as the

“Moving forward, it will be important to construct cohorts with greater racial and ethnic diversity...”

association of pLoFs in *EGLN1*, which encodes egl-9 family hypoxia-inducible factor 1, with both hemoglobin and hematocrit), indicating the tremendous value of quantitative traits for a gene burden-based PheWAS approach.

The approach of Dewey *et al.* and Abul-Husn *et al.* also demonstrates how analysis of dense genomic data from a single large regional health system can be leveraged for pedigree reconstruction. As was illustrated in DiscovEHR, 48% of participants had first-

degree (parents, siblings, children) or second-degree (such as grandparents, aunts, uncles, nieces, nephews) relatives in the data set—a result facilitated by close familial relationships in this stable population. Given the challenge of prospectively recruiting extended pedigrees, the advantages of “backing into” such pedigrees through genotype-first approaches and relating genotype to phenotype are clear. The availability of a large number of sequenced and phenotypically annotated patients also facilitates rapid recruitment into clinical trials of new therapeutics targeted to subsets of patients defined by genotype and/or subphenotype of disease.

Furthermore, applying WES to a large EHR phenotype database provides the potential for a “genotype-first” determination of the true population prevalence of a condition and thus the

identification of cases for early therapeutic intervention. Abul-Husn *et al.* identified 229 participants from the DiscovEHR cohort with evidence of pathogenic variants in genes known to cause familial hypercholesterolemia, representing a population prevalence of 1:222. As a comparison, algorithms validated to predict familial hypercholesterolemia from combinations of clinical factors identified only 24% of these cases. Notably, the majority of these individuals were not on appropriate low-density lipoprotein-lowering therapy. If this approach were applied broadly in the clinical setting, it could address the underdiagnosis of this disease (8, 9) and may lead to early intervention and reduced cardiovascular risk in more familial hypercholesterolemia patients.

The application of WES to an EHR-based cohort capitalizes on the phenotypic diversity of the underlying population, allowing the resulting genomic data to be used to address questions about the full spectrum of health- and disease-related traits. As illustrated by Dewey *et al.* and Abul-Husn *et al.*, continuous traits (such as weight, blood pressure, cholesterol, and glucose, which span a range of numerical values) can be extracted from structured numerical fields, whereas binary traits (such as diabetes, peripheral artery disease, or glaucoma, for which an individual either expresses the trait

or not) can be reconstructed from structured billing codes that indicate assigned diagnoses and performed procedures in the medical record. Much of the richness of the EHR, however, resides in the free text of the encounter notes and reports, and emerging natural language processing platforms should allow more sophisticated extraction and curation of this information.

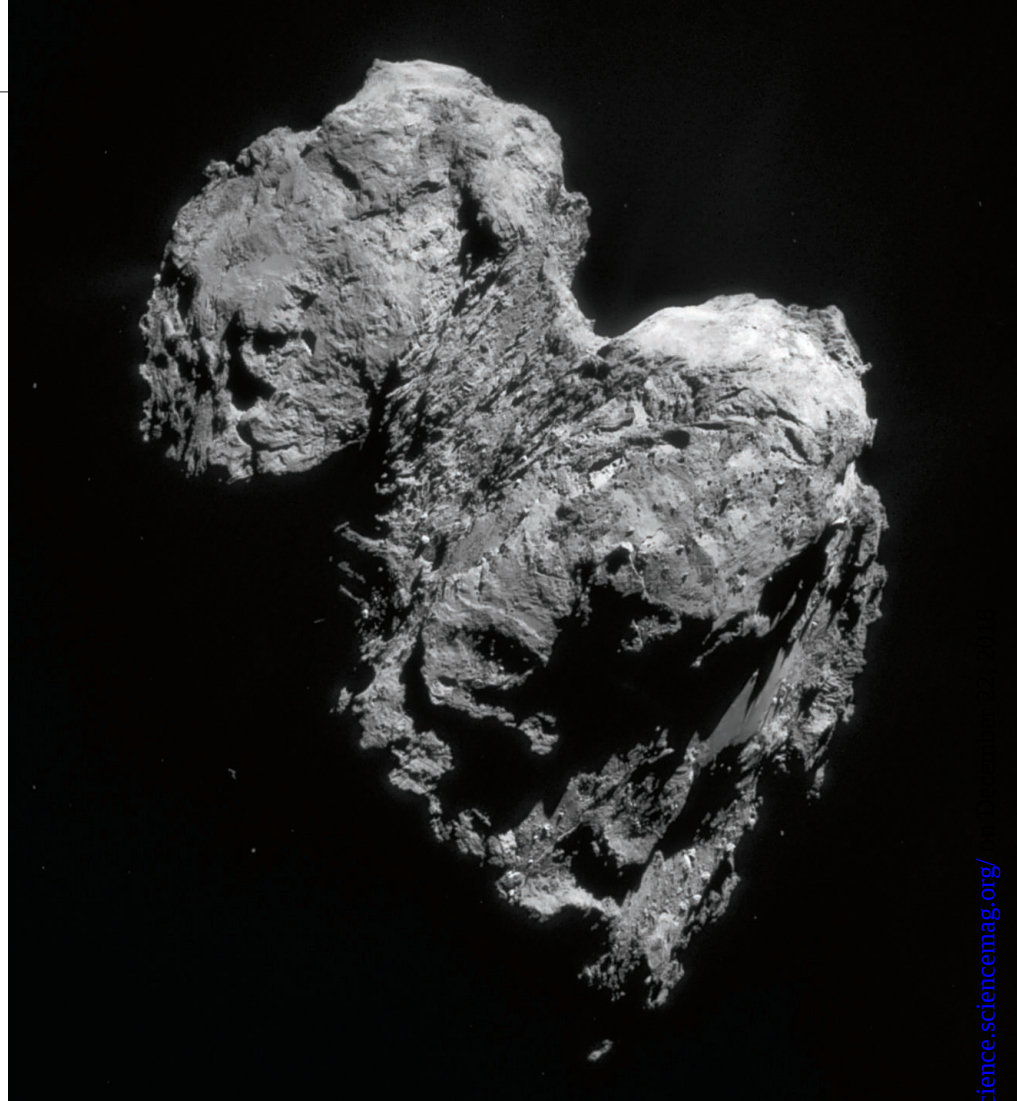
Even with the advantages of coupling dense rare variant genomic data with EHR-based phenotyping, there are several caveats that must be considered. The absence in the EHR of a phenotype for any given individual does not mean that the participant lacks the trait; it may have never been tested for, or the participant may have sought care outside of the health system captured by that EHR. In addition, subject enrollment in an EHR cohort reflects the underlying catchment of the health system. In this regard, it is important to note that the population in DiscovEHR was >98% white and only ~1% African American. Moving forward, it will be important to construct cohorts with greater racial and ethnic diversity, as has been accomplished in the initial efforts of the U.S. Department of Veterans Affairs' Million Veteran Program (10) and is aspired to in the proposed U.S. National Institutes of Health's "All of Us" cohort (11, 12).

Continued advances and price reductions in next-generation sequencing will make WES, and ultimately whole-genome sequencing, feasible on an increasingly larger scale. Better annotation of rare coding (and noncoding) variants and new statistical approaches will be critical to the analyses of these large data sets. Efforts to apply natural language processing and machine learning to EHR data will reveal substantially improved phenotypes and subphenotypes for analysis. These endeavors will require new experimental, computational, and statistical techniques to maximize biological discovery. With the appropriate investment, these undertakings will provide new insights into the genomic basis of health and disease and pave the way for the application of precision medicine on a population health scale. ■

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10.1126/science.aal4573



PLANETARY SCIENCE

More than a day in the life of a comet

Learning how a comet evolves can reveal the nature of the early solar system

By N. Dello Russo

In the past many humans regarded the appearance of a comet as an omen or harbinger of doom, but we now know them as time capsules from the formation of the solar system. Created from the gradual accretion of rocks, dust, and ice, comets have been preserved in the "deep freeze" of space far from the Sun, likely retaining in the present much of their formative character. The present-day struc-

ture and composition of comets provide a window into the conditions that prevailed during the birth of our solar system. Comet 67P/Churyumov-Gerasimenko, the target of the Rosetta mission, is a member of the Jupiter-family dynamical group of comets that have likely spent most of the last few billion years stored in the ring-shaped disc of icy bodies beyond the orbit of Neptune known as the Kuiper Belt. Subtle gravitational interactions can eventually push icy bodies in the Kuiper Belt reservoir toward the Sun where, under the gravitational influence of Jupiter, they evolve into short-period orbits around the Sun. Once its journey into the inner solar system begins, however, its

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PHOTO: ESA/ROSETTA/NAVCAM

Grayscale photograph of comet 67P/Churyumov-Gerasimenko taken by the Rosetta spacecraft.

days are numbered as heating from the Sun erodes the comet in a generally gradual but occasionally dramatic fashion. On pages 1563 and 1566 of this issue, Filacchione *et al.* (1) and Fornasier *et al.* (2), respectively, report on the long-term and close-up surveillance of 67P by Rosetta, revealing for the first time both the day-to-day and seasonal evolution of a comet nucleus (1, 2).

Comets contain fingerprints of the early solar system, but recognizing those signatures is difficult. The effects of evolutionary changes from radiation processing during billions of years in cold storage followed by recent extreme heating from many close solar passages must be recognized and disentangled from natal characteristics in order to read the primitive record of the early solar system. Until recently, the nature and properties of comet nuclei were based largely on inferences with little direct observational information. The typically several kilometer-sized nucleus of a comet is only directly observable from Earth when it is inactive and far away and thus faint and difficult to characterize. At closer distances, the Sun activates the release of large quantities of gas and dust forming a coma (or atmosphere) that shrouds the nucleus, allowing only the inference of nucleus characteristics from the properties of coma gas and dust.

The situation improved once spacecraft designed to study a comet's nucleus got up close and personal, piercing the coma to reveal unprecedented detail about nucleus surface morphology and the near-nucleus coma environment (3–7). But, as revolutionary as these missions were, they occurred during fleeting high-speed encounters, leaving important gaps about the life cycle of comets that snapshots in time cannot reveal. We know from Earth-based observations that comet evolution is not always steady but is punctuated by bursts of activity that cause sudden, often dramatic, brightening (8), occasionally shattering a comet to pieces (9), and sometimes even resulting in catastrophic disintegration (10). Rosetta was transformational because it was able to study the evolution of the nucleus up close over a 2-year period beginning with a phase of near inactivity, following it through its closest approach to the Sun when activity is highest, and staying with it as it retreated from the Sun and once more headed to dormancy (see the figure).

Rosetta was particularly well equipped to characterize comet surface morphology and track changes with time. Using a combination of spectroscopic and imaging systems spanning visible to infrared wavelengths, it

could reveal the composition of ices on the nucleus through detection of diagnostic spectral signatures (1) and the extent of ice and dust coverage on the nucleus through changes in surface color (2). A surprising result was the detection of volatile carbon dioxide ice (dry ice) in a surface region just coming out of the shadow of a 4-year winter while there was an absence in the same region of water ice, which is by far the dominant ice in comets (1). However, sheets of mixed water ice and dust existed over large portions of the nucleus just below a dusty surface (2). This compositional structure in the upper layers of the nucleus evolved as the comet approached the Sun, stripping off the dust layers to temporarily reveal an ice-rich surface before sublimation removed the ice, leaving again a dusty surface as 67P receded from the Sun (2).

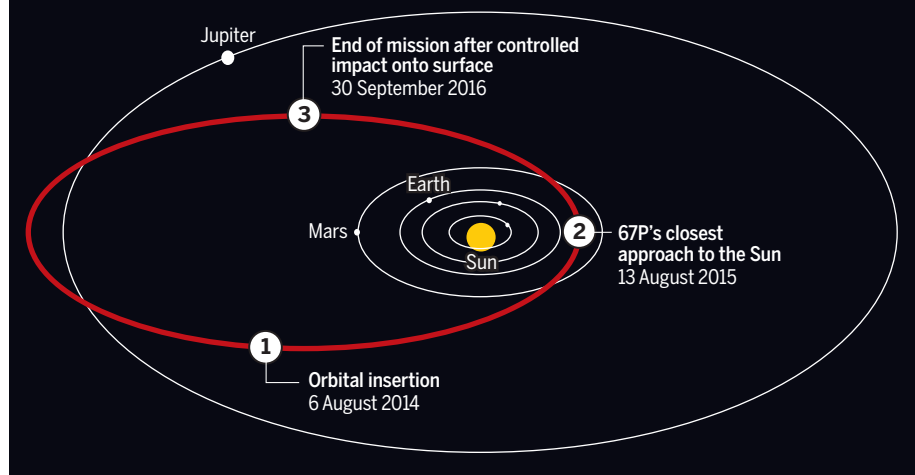
The temperature of the upper layers of the nucleus, as controlled by both the daily

surface erosion and volatile transport drive the evolution of 67P.

Rosetta has provided fundamental insights into the importance of evolutionary processes in comets. We now have direct evidence about how the composition and structure of the near-surface layers are driven by both diurnal and seasonal evolution. That volatile ices such as carbon dioxide still exist in comets today supports the idea that the interior of the nucleus still retains much of its natal character. Rosetta represents a major step forward in comet science, but many questions remain. Comets are compositionally diverse, and the few comets visited by spacecraft have remarkable variation in surface morphology, so it is unclear that the processes that drive evolution on 67P are universal. What is clear is that results from Rosetta will keep comet scientists hard at work trying to understand this diverse and evolving family of objects

To rendezvous and track

The Rosetta mission followed comet 67P for over a 2-year period covering a large portion of its orbit from near inactivity, through high levels of activity near closest approach to the Sun, and back out again. The orbit of comet 67P is shown in red in relation to the orbits of the five innermost planets. Mission milestones and extent are illustrated.



and seasonal exposure to sunlight, largely dictates the transportation and sequestration of ices in 67P. Daily variations were also observed as regions rotating in and out of shadow caused a cycle of frost condensation and sublimation on the surface in an ongoing interaction between the nucleus and coma (2). Over longer time scales, regions in shadow cool the surface to very low temperatures, whereas thermal inertia keeps interior layers at higher temperature, allowing the more volatile carbon dioxide to reach the colder surface from an interior reservoir before condensing, whereas water condenses deeper in the nucleus before it can reach the surface. These processes of

containing the most primitive material from the birth of our solar system—at least until the next comet mission provides new glimpses that both amaze us and dramatically alter our perceptions. ■

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10.1126/science.aal1964



BOOKS *et al.*

TECHNOLOGY POLICY

Tomorrow's arsenal

Two authors probe the technologies transforming warfare

By **Nayef Al-Rodhan**

From everyday life to the expansion of empires, technology has accompanied individuals and served to anchor geopolitical power. New technologies, however, are changing standard operating procedures due to their subtler and boundless capacity for surveillance. In warfare, they are generating the unprecedented potential for remote engagement, distancing attackers from the attacked.

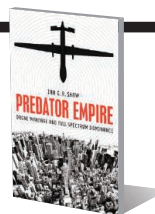
Ian Shaw's *Predator Empire* is a provocative analysis of the outreach of technology, specifically drones, as new tools to entrench U.S. power globally. The "Predator Empire" (so named by Shaw after Predator drones), aims at "full spectrum dominance": the control of all physical domains—terrestrial, maritime, and atmospheric. We are, in Shaw's opinion, "sleepwalking into totalitarianism," because the Predator Empire is, in essence, a "rule by nobody": tyrannical but without one discernible tyrant. It is a rule by "technics."

The technological power of the Predator Empire can be suffocating. For those living under drone-dotted skies, it can be traumatizing. So common is the trend toward unmanned surveillance that the drone is bound to become a brand of state power, "as recognizable as Coca Cola," Shaw maintains.

Shaw forebodingly reflects on this predicament but overstates the hopelessness of the situation, disregarding potential resistance. Calls for accountability show that we are not passively waiting for technology to crush our liberties. Amnesty International, for instance, has taken a public stance on encryption, call-

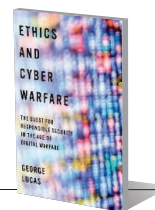
Predator Empire Drone Warfare and Full Spectrum Dominance

Ian G. R. Shaw
University of Minnesota Press,
2016. 334 pp.



Ethics and Cyber Warfare The Quest for Responsible Security in the Age of Digital Warfare

George Lucas
Oxford University Press, 2016.
201 pp.



ing it a matter of human rights (1).

Shaw argues that drones must be seen as geopolitical actors. There is merit to this argument because it provokes scholars to rethink the practical and theoretical role of technology. However, it must be challenged on technical grounds, because the technology on which drones are based allows for limited autonomy. Although they can infringe on people's space and affect their public lives, ultimately, they cannot—technically—take over.

Shaw's book also launches a larger critique of the all-encompassing electromagnetic spectrum, which mediates our surveillance. He believes that government surveillance has started to be legitimized too easily: Instead of being seen as a risk to democracy, it is increasingly seen as its savior. But many would argue that this is not an either/or question.

Like drones, cyberattacks are challenging traditional notions of warfare. George Lucas's *Ethics and Cyber Warfare* offers an eloquent, substantive, and original discussion of the main controversies and dilemmas related to the cyberdomain, including privacy and the legality of cyberwarfare.

Drones are changing the norms of modern warfare and dramatically enhancing the capacity for state-sponsored surveillance.

As is the case with drones, the perpetrators of cyberattacks are removed from the targeted physical location. This has often raised fears about the attribution of responsibility, the applicability of international law, and the threshold at which cyberattacks amount to acts of war.

Lucas cautions against the need to prepare for extreme scenarios. More plausible, he maintains, is the rise of state-sponsored hacktivism, operating with "weapons of mass disruption" that cause nuisances more than large-scale physical damage. He demonstrates that both ethics and existing laws can be effectively applied in cyberconflicts, despite significant gaps. The book also offers a meticulous exposé of ethical theories and examples that debunk some of the assumptions about cyberspace as a largely ungoverned space, where anything can happen.

In both tone and message, Lucas's book differs from Shaw's critique of the intrusive and repressive nature of late-20th- and early-21st-century technologies. For example, Lucas makes a compelling case that NSA surveillance, although morally problematic, will never replicate the atrocious Stasi-like surveillance programs of the 20th century. On the contrary, he contends, the intent of the U.S. government will always differ from that of an authoritarian regime.

This is a sensible takeaway: Technology does not exist above states or political agendas, but rather it is instrumental to their goals. The same thing could not be said about lone attackers, who are not bound by considerations outside their own moral compasses. Lucas suggests that a "code of ethics for cyber warriors" could be effective in limiting their attacks. However, power can be highly additive if unchecked. Given an environment where one can enjoy anonymity and no constraints, the thrill derived from such acts would likely outweigh a code of conduct (2).

Both of these books are valuable contributions to the literature, furthering relevant questions and raising still more. We should never be complacent about the trajectory of technology and especially technologies that can be immensely powerful tools of control. ■

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HISTORY OF SCIENCE

Divining science

A new tome argues that the ancient Babylonians deserve greater recognition in scientific histories

By Andrew Robinson

The eternal mystery of the world is its comprehensibility,” Albert Einstein famously mused in 1936. “The fact that it is comprehensible is a miracle.” The understandability of the natural world is all the more impressive when one considers the fact that fundamental human assumptions about time and space—the idea that there are 60 minutes in an hour, and that a circle can be broken down into 360 degrees—come from a time with “no articulated sense of nature ... no reference or word for it,” according to Francesca Rochberg, professor of Near Eastern studies at the University of California, Berkeley.

These concepts were borrowed by the ancient Greek philosophers from the mathematicians and astronomers of ancient Babylon, whose sexagesimal calculations first appeared in the cuneiform script—the world’s oldest writing system—in the Old Babylonian period, circa 2000 to 1600 BCE.

The Babylonians’ understanding of the heavens—including astronomical predictions—did not derive from any physical framework. They employed no classification of the Moon and planets as natural phenomena and conceived no physical laws of nature governing those bodies’ cyclical appearances. Neither had they any concept of a geometrical geocentric cosmos. On Earth, the cuneiform “determinative” sign for stone—a logogram today written as NA₄—denoted not merely natural stone but also beads manufactured from stone, from metals (gold and silver), and from shell. This incongruous variety of natural substances was classified under one sign because Babylonians perceived all as hard yet workable into artifacts.

Apart from the sexagesimal system, little else from the cuneiform world has been generally viewed as part of modern science, a view challenged by Rochberg. Throughout *Before Nature*, she argues that this pre-Greek

world deserves to be included in scientific history. “In doing so,” according to the book’s final sentence, “we will allow that our history of science can and should be inclusive of yet more variations on the scientific imagination.”

Rochberg argues that the cuneiform world’s preoccupation with divination, ritual, and incantation was motivated by a determination to establish “norms and anomalies within meaningful categories” and that this goal is inherently scientific. In a chapter entitled, “The Babylonians and the Rational,” she also argues that “magic” should not be



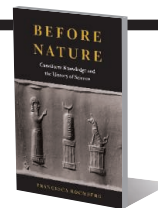
A clay model inscribed in Babylonian cuneiform maps the prognostications derived from each part of a sheep’s liver, 2050-1750 BCE.

used as a tool for separating science from nonscience because, in the cuneiform world, magic belonged neither to the natural nor to the supernatural. Three subsequent chapters develop these theses.

In “Causality and World Order,” Rochberg proposes that cuneiform scholars viewed nature as beholden to a set of laws. In “Observation of Astral Phenomena,” she maintains that the cuneiform world was capable of engaging systematically with astronomical phenomena. In “Prediction and Explanation in Cuneiform Scholarship,” she asserts that the cuneiform world’s emphasis on celestial divination reveals a commitment to prognostication and interpretation.

The underlying difficulty of the project emerges plainly from a well-known critique

Before Nature
Cuneiform Knowledge and
the History of Science
Francesca Rochberg
University of Chicago Press,
2016. 379 pp.



of Etruscan divination written by the ancient Roman philosopher Seneca and quoted twice by Rochberg: “While we believe that lightning is released as a result of the collision of clouds, they believe that clouds collide so as to cause lightning. For since they attribute everything to the gods’ will, they believe not that things have a meaning insofar as they occur, but rather that they happen because they must have a meaning.”

Seneca was both critical and respectful of Etruscan sophistication. This attitude provides a key, suggests Rochberg, to a proper present-day understanding of the cuneiform world’s view of divination. She cites a typical example of cuneiform divination known as extispicy, in which the entrails of sacrificial animals were used to make predictions: “If the gall bladder is turned and has wrapped around the ‘finger’: The king will seize the enemy country.” Here, omen and prognostication are linked by association or analogy and conform to the rule of inference, “if P, then Q.”

Links were not limited to conceptual or empirical categories but were often phonetic or semantic in nature. “If the coils of the intestine look like the face of Huwawa: it is the omen of the usurper king who ruled all the lands.” Here, “face of Huwawa” is written logographically as HUM.HUM, whereas “usurper king” is written phonetically as *hammā’u*, in a phonic echo.

Before Nature’s formidable erudition will fascinate cuneiformists while daunting nonspecialists and disturbing scientists, who will likely recoil from regarding divination as part of science. For noncuneiformists, the book’s most compelling parts will be its discussions of western civilization’s philosophical attempts to define “nature,” postdating the cuneiform world—from Aristotle to Einstein and his successors. “Real and independent as we may think nature and its orderliness are, the very notion of physical phenomena being subject to laws is a profoundly cultural claim, one that imparts a human value to the world external to human society,” argues Rochberg. ■

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LETTERS

Forest value: More than commercial

Edited by Jennifer Sills

IN THEIR RESEARCH Article “Positive biodiversity-productivity relationship predominant in global forests” (14 October, p. 196), J. Liang *et al.* establish a positive relation between forest productivity and commercial value to show the importance of tree species richness for supporting high productivity. They estimate that a drop in the current level of species richness in forests to one species would cost US\$166 to \$490 billion annually. However, species richness and ecological productivity are poor indicators for commercial value. According to the richness-value assumption, megadiverse tropical forests would provide high commercial value. In fact, the opposite is usually true. Cubbage *et al.* (1) showed that species-rich subtropical or tropical native forests are much less profitable than planted monocultures. In these forest types, only 20% of tree species or fewer are commercially valuable, and the harvests may be as low as 0.7 cubic meters per hectare per year (2), less than 10% of global average productivity according to Liang *et al.*

Planted forests provide a large part of the commercial value of the world's forests. The area of planted forests expanded from 167 to 278 million ha between 1990 and 2015, contributing 46% of the world's industrial roundwood in 2012 (3). The most profitable planted forests are not necessarily species rich (4), although forest types with two or more species may compete with monocultures in the temperate zone (5). For example, in South America, 88% of planted forests consist of introduced exotic species, usually grown as monocultures.

Such forest types show very high economic return (6). There is no economic evidence for the loss in commercial value that Liang *et al.* claim.

Postulating a positive relation between tree species richness and commercial value could potentially have adverse environmental consequences. For example, concluding that megadiverse tropical forests have innate commercial value would make it unnecessary to supplement this supposed value with rewards for landowners who preserve their native forests. Landowners might then continue to convert such forests to profitable monocultures such as eucalyptus, which have real commercial value. Species-rich forests indeed have an extremely high conservation and ecosystem service value, but their commercial value is often low.

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10.1126/science.aal2499

Response

PAUL AND KNOKE address the commercial value and profitability of forest biodiversity, which differs fundamentally from the economic value that we outlined in our Research Article. To translate the biophysical productivity gains from increased forest biodiversity into gross economic value, we used two different global estimates of forest

Eucalyptus monocultures are often more profitable than species-rich forests.

value: One estimate calculated the economic value of ecosystem services as a function of standing forest biomass (1); the other estimate determined the gross value added to the global forestry sector (2). Neither of these estimates directly reflects the commercial value of forests. Our estimates pertain to the sole contribution of tree species diversity, as it exists today, to global forest productivity, from which the economic value accrues. Our analysis—which includes nonmarket values not commonly captured in commercial forestry but excludes the contribution of forest biodiversity to carbon sequestration, wildlife habitat, and aesthetic and cultural values—reflects only a small portion of the true economic value of forest diversity at a global scale.

Our Research Article emphasized that biodiversity begets productivity value in either “unmanaged or extensively managed forests.” We did not compare unmanaged forests to planted monocultures. Monoculture profitability of planted forests results mostly from choosing a high-value and often exotic species that may be more commercially profitable in the short term because of lower costs of production, processing, and distribution; higher price per unit sold; and greater productivity associated with the unique traits of that species. We focused purely on biophysical productivity. Because market prices often fail to reflect the nontradable benefits associated with ecosystem services, market profitability is a flawed indicator of the true economic value of standing forests, as it is of most environmental amenities (3–5). Furthermore, the conversion of species-rich native forests to monoculture plantations of exotic species is quite different from the comparison of forests with current levels of diversity to hypothetical forests of one species; our Research Article used the latter comparison to strictly estimate the contribution of diversity to productivity in native forests. Learning how to simultaneously maximize complexity, resilience, nonmarket values, and profitability is a central challenge for forestry (6).

From a policy/management perspective, the positive biodiversity-productivity relationship across the world's forests helps justify rewarding landowners for preserving or enhancing the diversity of their native or planted forests. Establishing the underlying biophysical relationship between species richness and productivity, and translating that into economic

terms, reveals where markets fail to fully capture the true long-term economic value of forests, as opposed to the short-term commercial value, and thus where conservation attention is most needed.

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10.1126/science.aal2612

Iran's science landscape in context

IN HIS IN DEPTH News Story "In Iran, a shady market for papers flourishes" (16 September, p. 1197), R. Stone reports on the sale of plagiarized papers among Iran's scientists. This issue must be interpreted in context; Iran's fledgling science community (1) should not be judged as if it is a well-established scientific community in a scientifically advanced country.

The revolutionary Iran came into existence with some 26 universities and 175,000 students in 1979 (2), when the country's published papers totaled less than 400 (3, 4). For the first two decades after revolution, Iran's scientists were mainly engaged with establishing the necessary scientific and technological infrastructure. Twenty years after the revolution, the number of papers published by Iranian scientists was still not

much more than 1000 (3, 4).

In 2000, when the number of publications began to increase dramatically (3, 5), Iran's scientists and the science policy-makers started to become cautious about the output of the research done by our scientists. In the relatively short time that Iran has increased its published science output, its number of established institutions and students, and every other aspect of science activity, we have not been successful enough in establishing a scientific community with an innate code of ethics ("Creating a culture of ethics in Iran," M. S. Rezaee-Zavareh *et al.*, Letters, 21 October, p. 296). Many of Iran's university faculty are not even aware of what is and is not allowed in the scientific community. The time scale of structural change in the scientific sphere of the country has been too short and its acceleration too high to develop the necessary scientific "soft infrastructure."

It was just 2 years ago that the Ministry of Science, Research, and Technology of Iran asked our universities and research institutes to establish internal committees on science ethics (6). There is now a group of university faculty running a website about plagiarism called "Professors Against Plagiarism" (7). In the past 2 years, universities have started to

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address cases of plagiarism. Investigations are lengthy, and if a faculty member is found guilty, the results could include the termination of his or her contract.

There are likely many more cases of plagiarism within our scientific community, even more than already revealed by articles such as Stone's story. I am confident that the growing scientific community of Iran will establish a strong community with a vibrant discourse and code of ethics to minimize this plagiarism in time (8). I urge the international community to help Iran's efforts to establish a healthy scientific community for the sake of the region and the world.

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TECHNICAL COMMENT ABSTRACTS

Comment on "Structural basis of histone H3K27 trimethylation by an active polycomb repressive complex 2"

Ying Zhang, Neil Justin, Jon R. Wilson, Steven J. Gamblin

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Full text at <http://dx.doi.org/10.1126/science.aaf6236>

Response to Comments on "Structural basis of histone H3K27 trimethylation by an active polycomb repressive complex 2"

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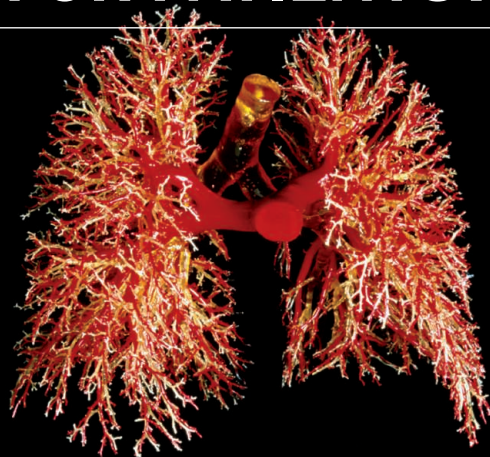
Full text at <http://dx.doi.org/10.1126/science.aaj2335>

ERRATA

Erratum for the Report "Local modulation of human brain responses by circadian rhythmicity and sleep debt" by V. Muto *et al.*, *Science* **354, aam5837 (2016).** Published online 23 December 2016; 10.1126/science.aam5837

Erratum for the Report "Coordination-induced weakening of ammonia, water, and hydrazine X-H bonds in a molybdenum complex" by M. J. Bezdek *et al.*, *Science* **354, aal4584 (2016).** Published online 9 December 2016; 10.1126/science.aal4584

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TECHNICAL COMMENT

TRANSCRIPTION

Comment on “Structural basis of histone H3K27 trimethylation by an active polycomb repressive complex 2”

Ying Zhang,* Neil Justin,* Jon R. Wilson, Steven J. Gamblin†

Jiao and Liu (Research Articles, 16 October 2015, aac4383) reported the crystal structure of the protein complex polycomb repressive complex 2 from *Chaetomium thermophilum*. This landmark structure has brought invaluable insights into the activation mechanism of this essential methyltransferase. However, the analysis of the x-ray data discussed below suggests that the description of oncogenic H3K27M peptide binding to the active site is incorrect.

Polycomb repressive complex 2 (PRC2) represses the expression of target genes through the methylation of associated nucleosomes on histone H3K27 (1). The catalytic subunit, EZH2, is a SET [su(var)3-9, enhancer-of-zeste and trithorax] domain lysine methyltransferase, which has a characteristic hydrophobic channel at its active site that positions the target substrate lysine side chain for methylation (2, 3). Mutations that disrupt the normal function of EZH2, both activating and deactivating, are associated with several cancer pathways (4, 5). In addition, the histone variant H3K27M is a dominant-negative somatic mutation that is implicated in high-grade pediatric gliomas (6, 7). Even low-level expression of this variant was found to effectively inhibit methylation of the wild-type histone by PRC2 (8). The recent structure of the catalytically active subcomplex of *Chaetomium thermophilum* PRC2, in complex with a H3K27M-derived peptide, provides a major breakthrough in understanding the basis of the potency of H3K27M and the disruption of normal EZH2 function (9). Unexpectedly, in the proposed model, the side chain of arginine-26, rather than the methionine at position 27, occupies the substrate “lysine” channel in the active site of the EZH2. We were surprised that the mutant side chain had no role in recognition and therefore reexamined the *C. thermophilum* x-ray data, Protein Data Bank (PDB) entry 5CHI.

The *C. thermophilum* EZH2 SET domain map features electron density that is characteristic of a protein chain at the active site in the groove where substrate peptide is expected to bind (2). In the Jiao and Liu model, the histone H3K27M

peptide (corresponding to residues 21 to 29) is built into this electron density (9). Surprisingly, there is no feature corresponding to the variant methionine-27 side chain in the electron density map. Previous biochemical data indicated that properties of the side chain at position 27 dictate the potency of peptide inhibitors (8, 10). These analyses indicated that an unbranched hydrophobic side chain is favored at the 27 position, and it is difficult to reconcile this with a model in which the methionine-27 side chain is not ordered. Further inspection of the *C. thermophilum* data highlights additional issues (Fig. 1A). Flanking side chains both show a poor fit to the electron density and have poor complementarity to the shape of the observed density, one of which forces the hydroxyl into a second hydrophobic pocket.

We propose an alternative interpretation of the *C. thermophilum* data. Inspection of the 2Fo – Fc and omit maps at each peptide side chain position suggested that the sequence LPGRGV would better fit the electron density and would be complementary to the binding surface (Fig. 1B). A search of the database entries with this sequence identified a perfect match in the *C. thermophilum* SUZ12 component of the PRC2 complex (residues 533 to 538) amino terminal to the VEFS [Vrn2-Emf2-Fis2-Su(z)12] domain. These residues are present in the crystallization fragment but were not accounted for in the atomic model. Furthermore, the molecular packing of the yeast PRC2 crystal suggests that this motif could be contributed from the SUZ12 subunit of a neighboring lattice molecule (Fig. 2). The LPGRGV sequence of SUZ12 is positioned between the VEFS domain and EZH2 on an artificial loop

that arises from the engineered linker between EZH2 C-terminus and the SUZ12 VEFS domain in the crystallographic construct. There are sufficient unbuilt residues to account for the distance from the N- and C-terminal ends of the LPGRGV motif to the neighboring molecule. Although it is likely that this sequence has lower affinity for EZH2 than the H3K27M peptide that was included for co-crystallization, the higher “effective” local concentration in the crystalline environment would allow it to out-compete the free peptide.

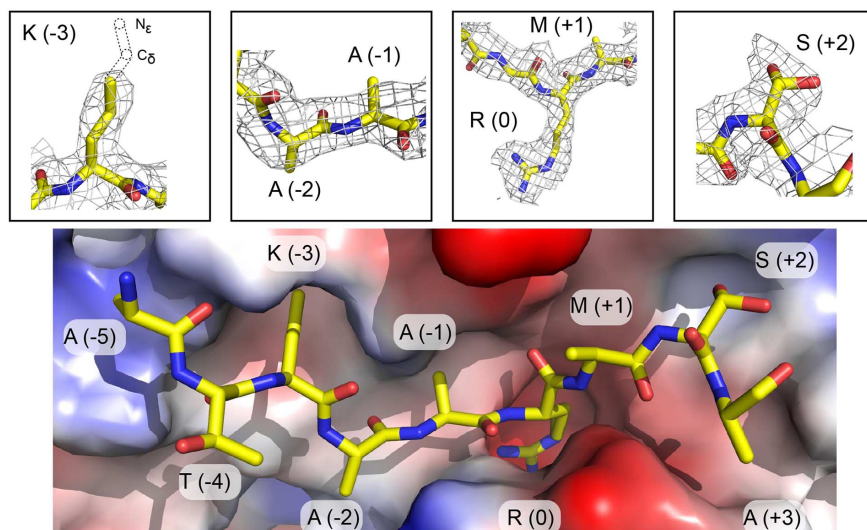
The binding mode reported for other SET domain structures with repressive peptides has the target lysine side chain occupying the substrate channel, but, notably, there is a conserved arginine in the –1 position, which has an important role in recognition (11–13). This arginine side chain sits in a complementary pocket on the surface of the SET domain and makes a series of salt bridges. This well-conserved arrangement was also observed in our recent structure of the human PRC2 complex with oncogenic H3K27M peptide (14). To support their assignment of the H3K27M peptide with noncanonical binding of the arginine, Jiao and Liu have presented activity data that shows that the R26A/K27M mutant peptide no longer inhibits methylation (9). Although consistent with their model, it is important to note that these data are also consistent with the binding mode found in other repressive peptide structures, where R26 makes essential contacts for peptide recognition. It would therefore be expected for the R26A/K27M to have reduced potency.

Our analysis therefore suggests that the proposed molecular model of the oncogenic histone peptide in the *C. thermophilum* PRC2 structure is incorrect, arising from the misidentification of the peptide bound to the SET domain. Thus, although we agree that there is an arginine side chain in the crystal structure of yeast PRC2, it is an artifact of the crystallization environment, and in physiological conditions the substrate channel would be occupied by the mutant methionine at position 27. This is important because it suggests that *C. thermophilum* PRC2 likely binds its histone substrate in a canonical manner. Perhaps more important, this reevaluation leads to a molecular basis for the potency of the H3K27M mutation that is consistent with earlier studies (6, 8, 10).

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A *C. thermophilum* PRC2 with H3 sequence (ATKAARMSA)**ACKNOWLEDGMENTS**

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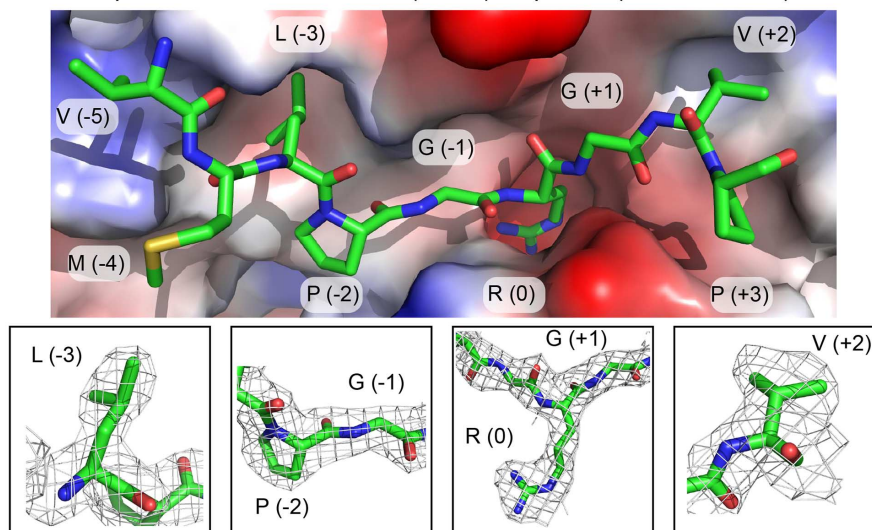
B *C. thermophilum* PRC2 with SUZ12 (VEFS) sequence (VMLPGRGV)

Fig. 1. Fitting of the H3 and VEFS sequence into the *C. thermophilum* x-ray data. (A) The side chains of the H3 sequence (KAARMS) do not fit well into the electron density map (refined $2F_o - F_c$ density). Residues K(-3) and M(+1) required truncation, and A(-1) is too large. To fill the observed density at the +2 position, a serine has been placed in two conformations. In addition, the electrostatic surface of the *C. thermophilum* EZH2 substrate binding cleft is not complementary to the H3 sequence—for example, requiring that K(-3) amine and the S(+2) hydroxyl would sit in hydrophobic pockets. The missing atoms for K(-3) are indicated by a dotted line. (B) The SUZ12 sequence (LPGRGV) is a better fit for the electron density map. The L(-3) and V(+2) side chains, in addition to fitting the density, are complementary to the two hydrophobic pockets. We have deposited coordinates for this alternative assignment of the substrate peptide with PDB code 5M5G.

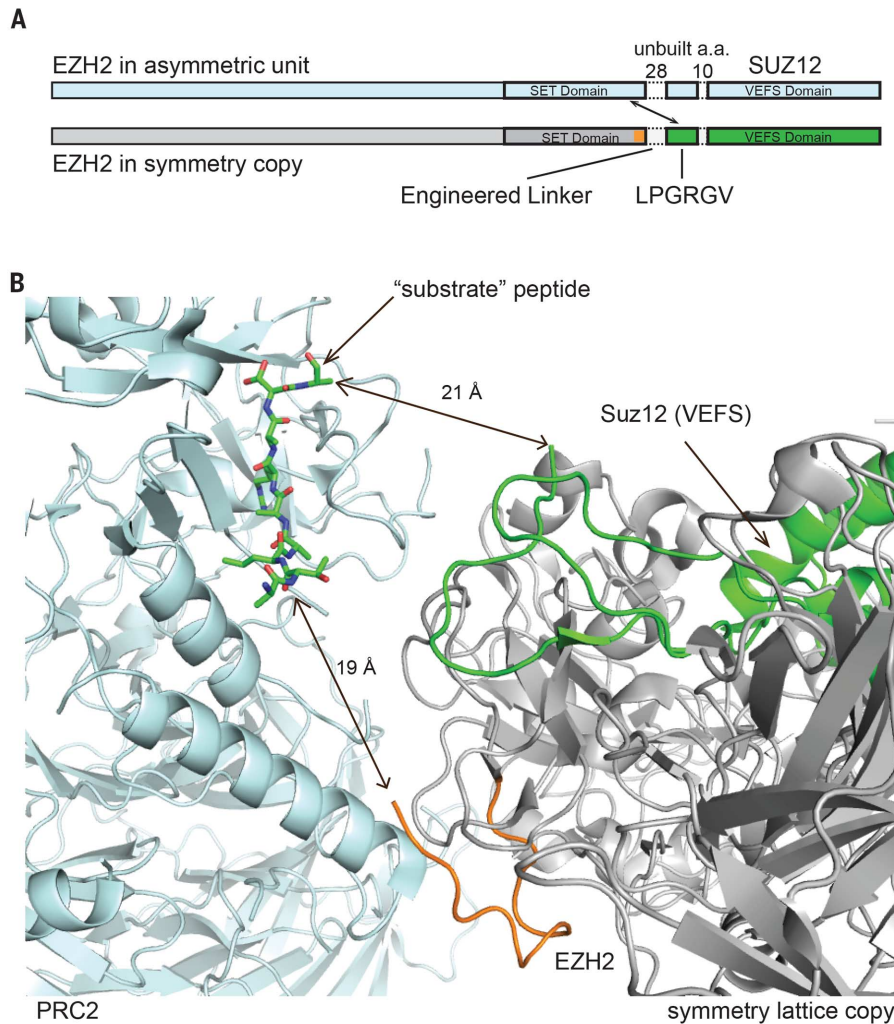


Fig. 2. The peptide chain occupying the SET domain active site most likely originates from a neighboring crystal lattice copy. (A) Schematic diagram of the EZH2-SUZ12 fusion construct in the asymmetric unit (blue) and symmetry related molecule (gray). The LPGRGV sequence is located N-terminal to the VEFS domain but immediately after an engineered loop on the crystallographic construct. The 28 residues preceding and 10 following this sequence are not built in the structure. (B) Cartoon representation of the relevant region of PRC2 (light blue) and a neighboring crystal lattice copy (gray). The SUZ12 protein is colored in green and the C terminus of the neighboring copy EZH2 in orange. The LPGRGV region is shown as a green stick representation in the active site of EZH2. The distance to the C terminus of the SET domain and the N terminus of the SUZ12 region is compatible with the number of unbuilt amino acids.

TECHNICAL RESPONSE

TRANSCRIPTION

Response to Comment on “Structural basis of histone H3K27 trimethylation by an active polycomb repressive complex 2”

Lianying Jiao and Xin Liu*

Zhang *et al.* suggested that in the crystal structure of a polycomb repressive complex 2 from *Chaetomium thermophilum* (ctPRC2), a flexible linker region, but not the H3K27M cancer mutant peptide, better fits the electron density. Based on our new data, we agree with this alternative interpretation and provide the crystal structure of ctPRC2 bound to bona fide H3K27M sequence.

Although displaying obvious sequence diversity, *Chaetomium thermophilum* polycomb repressive complex 2 (ctPRC2) is functionally and structurally similar to human PRC2 in both basal and stimulated states (1–3). In our initial crystallization efforts, the H3K27M cancer mutant peptide, which also inhibited the enzymatic activity of ctPRC2 in solution, was strictly required to generate ctPRC2 crystals of sufficient quality for structural determination (1). In addition, electron density that clearly corresponded to a short peptide was observed within the substrate-binding groove of the Ezh2 active site. We assigned the H3K27M peptide to the electron density primarily based on the arginine residue unambiguously found to occupy the lysine access channel, although we also noted that the mutated methionine residue was not fully resolved. However, according to Zhang *et al.*, a linker region located at the N terminus of the VEFS [Vrn2-Emf2-Fis2-Su(z)12] domain of Suz12 from a neighboring asymmetric unit in the crystal lattice better fits the electron density. Our own reexamination of the electron density map prompted by Zhang *et al.* (4) also indicated this possibility.

To clarify this situation and, more important, to reveal the mechanism of H3K27M recognition by ctPRC2, we replaced the corresponding linker region with an H3K27M peptide sequence in the context of the same Ezh2-Suz12(VEFS) fusion protein that was used for crystallization previously (Fig. 1). We obtained crystals of the sequence-modified ctPRC2 in complex with S-adenosylmethionine (SAM) and the H3K27me3-stimulating peptide in a similar crystal lattice under the same crystallization condition. We were not able to generate crystals of sufficient quality when SAM was replaced with S-adenosyl-L-

homocysteine (SAH). We determined the new crystal structure to 2.95-Å resolution, which showed that a methionine but not an arginine residue in the replaced H3K27M sequence from a neighboring asymmetric unit was inserted into the active site, contacting SAM directly (Fig. 1).

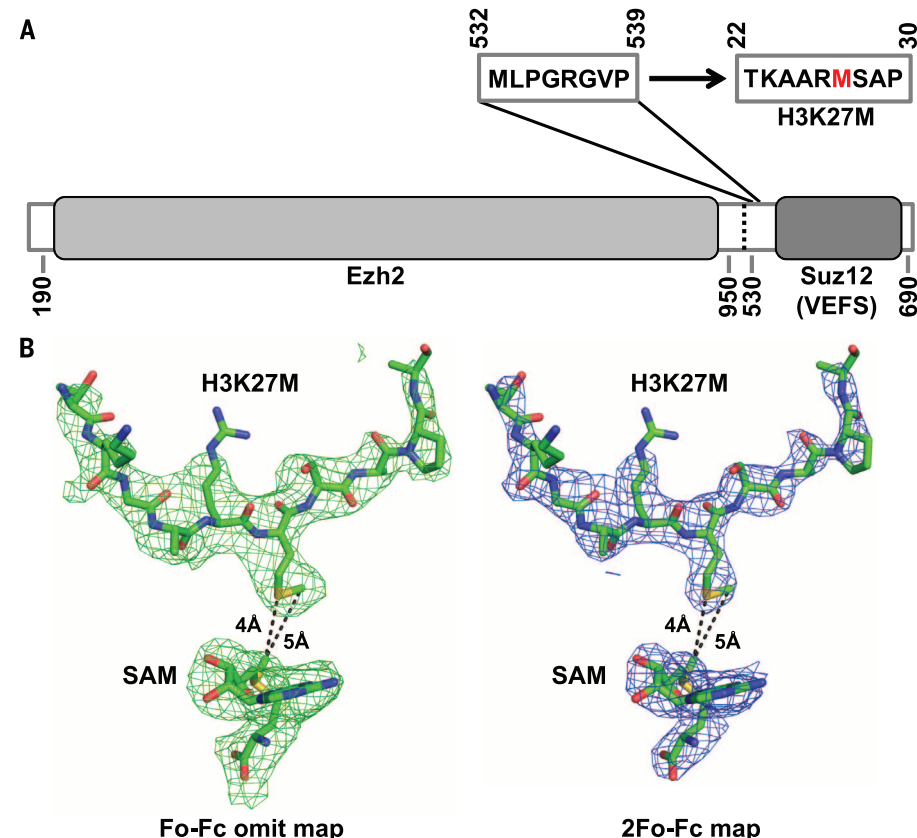


Fig. 1. Crystal structure of ctPRC2 bound to H3K27M. (A) Residues 532 to 539 of Suz12(VEFS) are replaced by residues 22 to 30 of histone H3K27M. Ezh2 and Suz12(VEFS) are indicated by gray boxes. (B) The Fo – Fc omit (left) and 2Fo – Fc (right) electron density maps contoured at 2.0 σ and 1.0 σ , respectively, that correspond to H3K27M and SAM, are indicated by the green and blue meshes. Structural models are shown in sticks. The side chain of the mutated methionine residue in H3K27M is clearly defined to occupy the lysine access channel and contact SAM.

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Only the aliphatic portion of residue R26 side chain is ordered under the current condition. The remainder of the structure remained essentially identical to the published ctPRC2 structure in the stimulated state. H3K27M thus appears to use the same structural mechanism to inhibit ctPRC2 catalysis as previously suggested for human PRC2 (2, 5).

The new crystal structure of SAM and H3K27M-bound ctPRC2 in the stimulated state was deposited in the Protein Data Bank under PDB accession code 5KKL. We also updated the previously deposited PDB entries 5CH1 and 5CH2 to 5KJH and 5KJI, respectively. All other results and analyses of our original publication remain unaffected. We apologize for any confusion that this misinterpretation may have caused.

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Coastal Maine Botanical Gardens provided a backdrop for science–religion discussions.

AAAS reaches out to theology students

Program fosters dialogue between scientific and religious communities

By **Michaela Jarvis**

Many Americans turn to religious leaders with questions about science and its implications, yet clergy members often have little exposure to science in their training. AAAS has taken the lead, based on years of planning, in addressing this conundrum by organizing the Science for Seminaries program, with a pilot project launched in 2013. The pilot effort provided science resources for seminaries as they sought to equip future religious leaders with solid scientific information and with connections to scientists.

The initial results are appreciable. One seminary professor, Bill Brown, reported a “dramatic rise” in students’ appreciation of science, describing activities associated with the project as “transformative.”

Brown, the William Marcellus McPheeters Professor of Old Testament at Columbia Theological Seminary in Decatur, Georgia, elaborated on the most recent campus science events undertaken as part of the project. “We’ve had the best attendance yet among our students, followed by both formal and informal conversations...In short, it seems that a sense of wonder about science is being cultivated on campus.”

To most effectively connect with seminaries, AAAS partnered with the Association of Theological Schools, an accrediting association for graduate schools that train clergy. The pilot project, involving 10 seminaries representing a wide variety of Christian religious traditions, was designed to help professors incorporate relevant science into at least two of each seminary’s core courses. The participating schools also set out to organize at least one campus-wide event each to explore the relevance of science to theological education.

By the most recent count at the close of the pilot project, more than 116 courses across the pilot schools had been revised, and at

least 77 related events took place. Similarly, 137 applicants vied for 37 spots at related summer retreats designed to engage new seminary professors beyond those from the first 10 pilot schools. Perhaps most importantly, the project brought practicing scientists and seminary professors together to sculpt the most effective ways of conveying scientific advances and relevance to students.

“We were pleasantly surprised by the wide range of science topics that seminaries chose to explore, including cosmology, genetics, neuroscience, paleontology, and more,” said Jennifer Wiseman, the director of the AAAS Dialogue on Science, Ethics, and Religion (DoSER) program, which led the effort. “We were also pleased by the large number of scientists who were eager to get involved with the program, by, for example, giving guest science lectures in seminary classrooms and developing relationships with seminary professors.”

Last summer’s retreats brought together pilot project faculty and scientists with representatives of additional theological schools, providing a venue for sharing insights and strategies. After being introduced to Science for Seminaries at one of the retreats, Beth Rath, an assistant professor of philosophy, went back to Borromeo Seminary and offered a class called “What Does Science Prove?: Topics at the Intersection of Science and Religion.”

One of the students who took the class, Andrew Karpinski, said it helped him develop a new understanding of the science of evolution, which he had learned in high school, though feeling somewhat dissatisfied with the totally secular discussion offered in school.

“If you incorporate science into your religious education, you get the fullest picture of the world we live in,” said Karpinski, an aspiring religious leader who said he feels ready to incorporate science into his work with young people in inner-city Cleveland.

Rath said Karpinski was among many in the class who came away with new insights.

"This was the first time I've ever offered the course," Rath said, "and it has been hugely successful. The impact that the class made on the students was more than I had hoped for." Rath added that the retreat she attended put her in touch with neuroscientist Nancy Adleman, of the Catholic University of America, who helped Rath incorporate scientific articles into a unit on free will and moral responsibility.

Others who attended have also begun incorporating science into their courses. Jim Higginbotham, associate professor of pastoral care and counseling at Earlham School of Religion, in Richmond, Indiana, has prepared a class on death and dying that will ask students to address the bioethical issues related to the end of life, to "create a critical dialogue" between the natural sciences and spirituality. Grace Kao, associate ethics professor at Claremont School of Theology, in Claremont, California, mentioned additions she will make to her Introduction to Christian Ethics course, such as discussing epigenetic alterations associated with war trauma for a session on war and peace, the science behind shopping and the ways that poverty can change your genes for a segment about economics, and an exploration of whether genes can predict a person's liberalism and conservatism for a session on religion and politics. Steven Studebaker, associate professor of systematic and historical theology and the Howard & Shirley Bengal Chair in evangelical thought at McMaster Divinity College in Hamilton, Ontario, is planning on including in his Protestant theologians class John Polkinghorne, physicist and Anglican priest, and Philip Clayton, philosopher of religion and science.

"The activities of the retreat cast a vision for what integrating science in the seminary classroom can look like," Studebaker said.

As students at the 10 seminaries who participated in the 3-year pilot project experience the revised classes and campus-wide science-religion events, the schools continue to survey their reactions. The results have generally been extremely positive.

At Multnomah Biblical Seminary in Portland, Oregon, most students responded that they strongly agreed or agreed with statements such as "I recognize that scientific discoveries might have a bearing on how I approach life, work, and ministry," the surveys showed.

In April, a 2-day religion and science conference at Multnomah received rave reviews.

"The conference was a refreshing example of dialogue, rather than war, between faith and science," said Sara Mannen, Multnomah Master of Arts in Theological Studies student. "Recognizing our shared values of awe at the universe and desiring the good of society helped provide me with a starting point for conversations concerning faith and science."

Almost all of the students at Wake Forest University School of Divinity, in Winston-Salem, North Carolina, said they had enjoyed science education opportunities including public lectures by scientists such as cosmologist John Barrow and science field trips such as to the Kennedy Space Center, said Associate Professor of Christian Ethics Kevin Jung.

Given the impact of the pilot Science for Seminaries project, DoSER is expanding the effort to assist seminaries, which is seen as an opportunity to affect public understanding and support for science—and the degree to which science can benefit society, a mission of AAAS.

"There were many more interested seminaries and seminary professors that we could not accommodate in this initial pilot project," said Se Kim, associate director of DoSER. "We'd like to provide science assistance for more seminaries and will also continue to help interested scientists get connected to seminaries interested in their scientific expertise."

In offshoots of the program, DoSER is now working with continuing education programs for active clergy at four institutions, and is assisting two Rabbinic training institutions with their efforts to incorporate science.

Meanwhile, as the pilot seminaries build upon their initial efforts, they are sharing with other seminaries resources they have developed so far, such as revised syllabi, via the project website: scienceforseminaries.org. Additionally, AAAS has produced a series of discussion-provoking films for classroom use, called "Science: The Wide Angle." The film series features 14 of the world's leading scientists and three historians of science discussing exciting scientific advances—and their own wonder and amazement as they explore our world.

As for enduring impact, Professor Jung quoted one of the Wake Forest students asked about the experience of exploring science as part of a religious education: "I think it will resonate for many years," the student wrote, "as I continue to dig deeper into the issues of my congregants."

More information about the Science for Seminaries program, and the "Science: The Wide Angle" films, are available at scienceforseminaries.org.

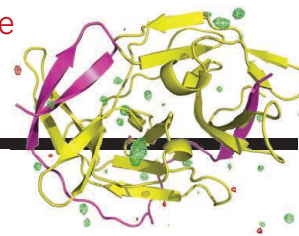


Seminary faculty and advisers chatted during a trolley ride to Bigelow Laboratory for Ocean Sciences.

RESEARCH

High-resolution crystal structures of the Zika virus protease

Zhang et al., p. 1597



IN SCIENCE JOURNALS

Edited by **Stella Hurtley**

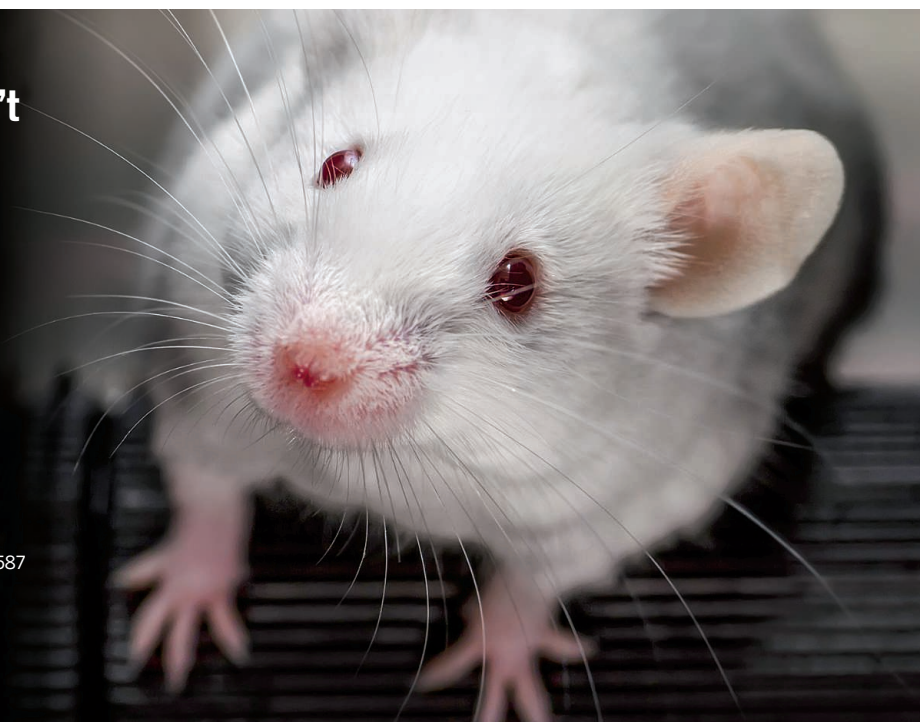
BRAIN RESEARCH

Now you feel it, now you don't

What determines the detection of a sensory stimulus? To address this question, Takahashi *et al.* combined in vivo two-photon imaging, electrophysiology, optogenetics, and behavioral analysis in a study of mice. Calcium signals in apical dendrites of pyramidal neurons in the somatosensory cortex controlled the perceptual threshold of the mice's whiskers. Strong reduction of dendritic calcium signaling impaired the perceptual detection threshold so that an identical stimulus could no longer be noticed. —PRS

Science, this issue p. 1587

Mice shed light on mechanisms involved in whisker sensitivity.



COMETARY SCIENCE

Rosetta observes sublimating surface ices

Comets are "dirty snowballs" made of ice and dust, but they are dark because the ice sublimates away, leaving some of the dust behind on the surface. The Rosetta spacecraft has provided a close-up view of the comet 67P/Churyumov-Gerasimenko as it passes through its closest point to the Sun (see the Perspective by Dello Russo). Filacchione *et al.* detected the spectral signature of solid CO₂ (dry ice) in small patches on the surface of the nucleus as they emerged from local winter. By modeling how the CO₂ sublimates, they constrain the composition of comets and how ices generate the gaseous

coma and tail. Fornasier *et al.* studied images of the comet and discovered bright patches on the surface where ice was exposed, which disappeared as the ice sublimated. They also saw frost emerging from receding shadows. The surface of the comet was noticeably less red just after local dawn, indicating that icy material is removed by sunlight during the local day. —KTS

Science, this issue p. 1563, p. 1566; see also p. 1536

TOPOLOGICAL MATTER

Watching Majorana bound states form

Majorana bound states (MBSs) are peculiar quasiparticles that may one day become the cornerstone of topological quantum

computing. To engineer these states, physicists have used semiconductor nanowires in contact with a superconductor. Although many of the observed properties align with theoretical predictions, a closer look into the creation of MBSs is desirable. Deng *et al.* fabricated nanowires with a quantum dot at one end that served as a spectrometer for the states that formed inside the superconducting gap of the nanowire. Using this setup, topologically trivial bound

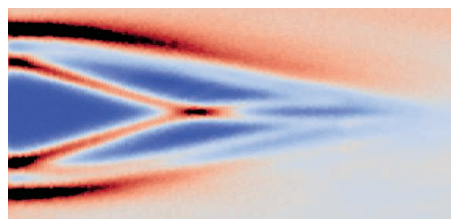
states were seen to coalesce into MBSs as the magnetic field was varied. —JS

Science, this issue p. 1557

STRUCTURAL BIOLOGY

Snapshots of bacteriorhodopsin

Bacteriorhodopsin is a membrane protein that harvests the energy content from light to transport protons out of the cell against a transmembrane potential. Nango *et al.* used time-resolved serial femtosecond crystallography at an x-ray free electron laser to provide 13 structural snapshots



Nanowires reveal an exotic state of matter.

of the conformational changes that occur in the nanoseconds to milliseconds after photoactivation. These changes begin at the active site, propagate toward the extracellular side of the protein, and mediate internal protonation exchanges that achieve proton transport. —VV

Science, this issue p. 1552

CATALYSIS

Boron nitride catalysis

Propene is one of the highest-volume organic chemicals produced. Propene has mainly been made from naphtha, but changes in the global supply chain are creating shortages. Direct conversion from propane, a component of natural gas, via reaction with oxygen is an attractive alternative, but existing approaches produce a large fraction of unwanted CO and CO₂. Grant *et al.* report that boron nitride, normally an unreactive material, has high selectivity to catalyze the production of propene (77%) and ethene (13%). —PDS

Science, this issue p. 1570

MIGRATION

Mass movement of “invisibles”

We know a lot about vertebrate migrations globally. However, the majority of animals that live on this planet are invertebrates, and we know very little about their movements. Hu *et al.* monitored the migration of large and small insects over the southern United Kingdom for a decade. They found that more than a trillion insects move across this region annually. The movement of such a large biomass has considerable impacts on the ecosystems between which the insects migrate. —SNV

Science, this issue p. 1584

PLANT SCIENCE

Prohormone processing by subtilases

A flower that has gone to seed will drop its petals in a regulated

process called abscission. Schardon *et al.* analyzed the production of the peptide hormone that regulates floral organ abscission in the model plant *Arabidopsis thaliana*. They used tissue-specific expression of proteinase inhibitors to identify the subtilisin-like proteinases that act as prohormone convertases required for peptide hormone production in plants. —PJH

Science, this issue p. 1594

IMMUNOLOGY

A balance between staying and leaving

Mobilization of neutrophils from the bone marrow is determined by the balance between two opposing chemokines that either keep neutrophils in the bone marrow or recruit them to tissues. Both chemokines activate the small guanosine triphosphatase Rac. Campa *et al.* found that the time that it took active Rac to return to baseline determined how long neutrophils stayed in the bone marrow. Mice lacking a Rac inhibitor had more neutrophils in the bone marrow and fewer circulating neutrophils than control mice had. —WW

Sci. Signal. **9**, ra124 (2016).

CANCER

Combining drugs as the doctor ordered

Cancer immunotherapy is being used for a growing number of cancers. Chemotherapy is still the mainstay of cancer treatment, however, and it can be difficult to find good ways to combine the two approaches. Mathios *et al.* systematically evaluated the effectiveness of local or systemic chemotherapy before or after immune checkpoint inhibition in mouse models of glioblastoma. Local chemotherapy was particularly effective in combination with checkpoint inhibition, whereas systemic chemotherapy was too damaging to the immune system for the combination to be useful. —YN

Sci. Transl. Med. **8**, 370ra180 (2016).

IN OTHER JOURNALS

Edited by **Sacha Vignieri**
and **Jesse Smith**

ICE SHELVES

Unwelcome rifts

Ice sheets have ice shelves, which continually break apart to form icebergs when the shelves grow too large for their environment. This is normal and typically occurs independently of the conditions of the ocean on which they float. However, a recent rifting event in the middle of the Pine Island Glacier of Antarctica seems to be the result of ocean forcing, report Jeong *et al.* This development is not a welcome one, because Pine Island Glacier appears already to have begun an irreversible retreat due to global warming, one of the effects of which is to increase ocean temperatures near many ice shelves. —HJS

Geophys. Res. Lett. 10.1002/2016GL071360 (2016).



NEUROSCIENCE

Tracking extracellular space in the brain

Extracellular space takes up a large percentage of the brain. Its size changes with the sleep-wake cycle but also during brain development and normal aging,

as well as under pathological conditions such as neurodegeneration. Godin *et al.* injected near-infrared luminescent carbon nanotubes into rat brains and tracked their diffusion in the extracellular space. This method revealed the dimensions of the extracellular space in live brain

ALSO IN SCIENCE JOURNALS

Edited by Stella Hurtley

HUMAN GENETICS

Unleashing the power of precision medicine

Precision medicine promises the ability to identify risks and treat patients on the basis of pathogenic genetic variation. Two studies combined exome sequencing results for over 50,000 people with their electronic health records. Dewey *et al.* found that ~3.5% of individuals in their cohort had clinically actionable genetic variants. Many of these variants affected blood lipid levels that could influence cardiovascular health. Abul-Husn *et al.* extended these findings to investigate the genetics and treatment of familial hypercholesterolemia, a risk factor for cardiovascular disease, within their patient pool. Genetic screening helped identify at-risk patients who could benefit from increased treatment. —LMZ

Science, this issue p. 1549, p. 1550

CELL FATE DECISIONS

How to grow hair or sweat glands

Unlike other mammals that must pant or seek shade or water when overheated, humans are able to self-cool to tolerate extreme heat. Sweat glands, which enable humans to run in marathons, are instrumental for this remarkable feat. Lu *et al.* investigated skin appendage diversity during development of the furry backs and sweaty paws of mice (see the Perspective by Lai and Chuong). They also examined human skin, which is capable of making both hairs and sweat glands in the same area of the body. Epithelial-mesenchymal interactions, with varied signaling pathways that act at specific times in development, are key to producing different skin appendages for adaptation to the environment. —BAP

Science, this issue p. 1551;
see also p. 1533

QUANTUM ELECTRONICS

Extending qubit lifetime through a shaped environment

Qubits are the quantum two-level systems that encode and process information in quantum computing. Kept in isolation, qubits can be stable. In a practical setting, however, qubits must be addressed and interact with each other. Such an environment is typically viewed as a source of decoherence and has a detrimental effect on a qubit's ability to retain encoded information. Gustavsson *et al.* used a sequence of pulses as a source of "environment shaping" that could substantially increase the coherence time of a superconducting qubit. —ISO

Science, this issue p. 1573

FISHERIES

Marine benefits of the Paris Agreement

Keeping recent global agreements to limit temperature increases to 1.5° to 2°C above preindustrial levels will have benefits across terrestrial ecosystems. But what about marine ecosystems? Cheung *et al.* modeled the influence of temperature increases on two key measures of fishery sustainability, catch and species turnover (see the Perspective by Fulton). Limiting temperature increases to 1.5°C substantially improved catch potential and decreased turnover of harvested species. These results provide further support for meeting this important goal. —SNV

Science, this issue p. 1591;
see also p. 1530

QUANTUM OPTICS

A quantum optical circulator

A circulator is a passive three- or four-port device that routes signals according to a

simple protocol: If the ports are numbered in ascending order, a signal that enters the circulator through port 1, 2, 3, or 4 exits it through port 2, 3, 4, or 1, respectively. Scheucher *et al.* demonstrate an integrated optical circulator that operates by using the internal quantum state of a single atom (see the Perspective by Munro and Nemoto). Moreover, the routing can be reversed by flipping the atomic spin. Such an integrated optical device may be important for routing and processing quantum information in scalable integrated optical circuits. —ISO

Science, this issue p. 1577;
see also p. 1532

NANOMATERIALS

Probing packing rules

The crystals of a well-defined ligand-covered gold nanoparticle can reveal how packing into a lattice happens. Zeng *et al.* synthesized nanoparticles with a 246-atom gold core surrounded by 80 4-methylbenzenethiol ligands. These nearly spherical nanoparticles did not pack into a cubic arrangement but instead formed a lower-symmetry monoclinic structure. A hierarchy of interparticle ligand interactions controlled the packing, including sets of chiral packing arrangements that reversed between layers. —PDS

Science, this issue p. 1580

STRUCTURAL BIOLOGY

A closed conformation for Zika virus enzyme

The recent Zika virus epidemic highlights the need for antiviral drugs. One important drug target is the virus's NS2B-NS3 protease, an enzyme that is critical for viral replication. Zhang *et al.* report high-resolution crystal structures of the protease as a free enzyme and with a peptide bound to the active site in the reverse position. The structures reveal that, unlike in other flavi-

viruses, the protease adopts a closed conformation, in which NS2B engages NS3 to form the empty substrate-binding site. Moreover, substrate binding did not substantially alter the conformation of the enzyme. —KLM

Science, this issue p. 1597

OCEANS

A precarious balance

All ocean basins have regions with almost no oxygen at depths of a few hundred meters. These oxygen minimum zones are growing in size, and many coastal regions are also devoid of oxygen owing to increased nutrient input from rivers. In a Perspective, Watson explains that in the geologic past, major environmental crises have caused the entire ocean to deoxygenate, with disastrous consequences for ocean life. Modern disturbances would have to continue for a thousand years for the entire ocean to be affected, but locally, today's growing dead zones are already lethal to animals. —JFU

Science, this issue p. 1529

RESEARCH ARTICLE SUMMARY

HUMAN GENETICS

Distribution and clinical impact of functional variants in 50,726 whole-exome sequences from the DiscovEHR Study

Frederick E. Dewey *et al.*

INTRODUCTION: Large-scale genetic studies of integrated health care populations, with phenotypic data captured natively in the documentation of clinical care, have the potential to unveil genetic associations that point the way to new biology and therapeutic targets. This setting also represents an ideal test bed for the implementation of genomics in routine clinical care in service of precision medicine.

RATIONALE: The DiscovEHR collaboration between the Regeneron Genetics Center and Geisinger Health System aims to catalyze genomic discovery and precision medicine by coupling high-throughput exome sequencing to longitudinal electronic health records (EHRs) of participants in Geisinger's MyCode Community Health Initiative. Here, we describe initial insights from whole-exome sequencing of 50,726 adult participants of predominantly European ancestry using clinical phenotypes derived from EHRs.

RESULTS: The median duration of EHR data associated with sequenced participants was

14 years, with a median of 87 clinical encounters, 687 laboratory tests, and seven procedures per participant. Forty-eight percent of sequenced individuals had one or more first- or second-degree relatives in the sample, and genome-wide autozygosity was similar to other outbred European populations. We found ~4.2 million single-nucleotide variants and insertion/deletion events, of which ~176,000 are predicted to result in loss of gene function (LoF). The overwhelming majority of these genetic variants occurred at a minor allele frequency of $\leq 1\%$, and more than half were singletons. Each participant harbored a median of 21 rare predicted LoFs. At this sample size, ~92% of sequenced genes, including genes that encode existing drug targets or confer risk for highly penetrant genetic diseases, harbor rare heterozygous predicted LoF variants. About 7% of sequenced genes contained rare homozygous predicted LoF variants in at least one individual. Linking these data to EHR-derived laboratory phenotypes revealed consequences of partial or complete LoF in humans. Among these were previously unidentified associations between

predicted LoFs in *CSF2RB* and basophil and eosinophil counts, and *EGLN1*-associated erythrocytosis segregating in genetically identified family networks. Using predicted LoFs as a model for drug target antagonism, we found associations supporting the majority of therapeutic targets for lipid lowering. To highlight the opportunity for genotype-phenotype association discovery, we performed exome-wide association analyses of EHR-derived lipid values, newly

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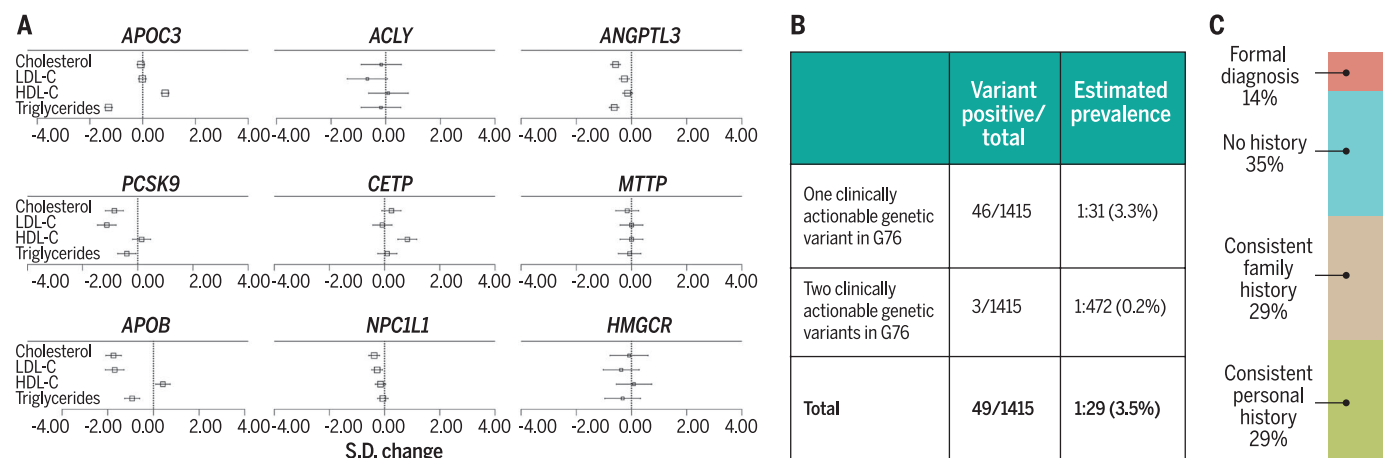
implicating rare predicted LoFs, and deleterious missense variants in *G6PC* in association with triglyceride levels. In a survey of 76 clinically actionable disease-associated genes, we

estimated that 3.5% of individuals harbor pathogenic or likely pathogenic variants that meet criteria for clinical action. Review of the EHR uncovered findings associated with the monogenic condition in ~65% of pathogenic variant carriers' medical records.

CONCLUSION: The findings reported here demonstrate the value of large-scale sequencing in an integrated health system population, add to the knowledge base regarding the phenotypic consequences of human genetic variation, and illustrate the challenges and promise of genomic medicine implementation. DiscovEHR provides a blueprint for large-scale precision medicine initiatives and genomics-guided therapeutic target discovery. ■

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Therapeutic target validation and genomic medicine in DiscovEHR. (A) Associations between predicted LoF variants in lipid drug target genes and lipid levels. Boxes correspond to effect size, given as the absolute value of effect, in SD units; whiskers denote 95% confidence intervals for effect. The size of the box is proportional to the logarithm (base 10) of predicted LoF carriers. (B and C) Prevalence and expressivity of clinically actionable genetic variants in 76 disease genes, according to EHR data. G76, Geisinger-76.

RESEARCH ARTICLE

HUMAN GENETICS

Distribution and clinical impact of functional variants in 50,726 whole-exome sequences from the DiscovEHR study

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The DiscovEHR collaboration between the Regeneron Genetics Center and Geisinger Health System couples high-throughput sequencing to an integrated health care system using longitudinal electronic health records (EHRs). We sequenced the exomes of 50,726 adult participants in the DiscovEHR study to identify ~4.2 million rare single-nucleotide variants and insertion/deletion events, of which ~176,000 are predicted to result in a loss of gene function. Linking these data to EHR-derived clinical phenotypes, we find clinical associations supporting therapeutic targets, including genes encoding drug targets for lipid lowering, and identify previously unidentified rare alleles associated with lipid levels and other blood level traits. About 3.5% of individuals harbor deleterious variants in 76 clinically actionable genes. The DiscovEHR data set provides a blueprint for large-scale precision medicine initiatives and genomics-guided therapeutic discovery.

The application of high-throughput DNA sequencing to human cohorts enables genetic discoveries spanning the development of comprehensive catalogs of rare and common genetic variations (1, 2) and genetic discoveries of previously unidentified Mendelian disease genes (3, 4) and implicates rare variants in common complex diseases (5–7). Identification of rare loss of gene function variants, or “human knockouts” (8–11), linked to epidemiological data (12) or phenotypes captured in structured research or clinical records (9, 10, 13, 14) can facilitate discovery of phenotypic associations that inform human biology and disease pathophysiology. Furthermore, sequencing efforts have identified new therapeutic targets (15–22), spurring the development of therapeutics for several diseases from lipid disorders to cancer. The implementation of

precision medicine requires further investigation of genetic factors that affect health and disease, the development of targeted therapeutics, and further understanding of the utility of genomics for clinical care.

In 2014, the Regeneron Genetics Center, a wholly owned subsidiary of Regeneron Pharmaceuticals, and the Geisinger Health System (GHS), an integrated health system in central and north-eastern Pennsylvania, initiated the DiscovEHR collaboration. DiscovEHR strives to elucidate genetic factors that affect a wide range of human diseases and related traits to unveil new biology and drug targets, as well as to scale the implementation of precision medicine by identifying, returning, and acting on clinically actionable genetic variants.

Results

Protein-coding variation in 50,726 exomes

The DiscovEHR cohort is derived from GHS patients who consented to participate in the Geisinger MyCode Community Health Initiative (23). MyCode participants consent to provide blood

and DNA samples for a system-wide biorepository for broad research purposes, including genomic analyses, and linking to data in the GHS electronic health record (EHR). MyCode participants agree to be recontacted for additional phenotyping and return of clinically actionable results to inform their health care. The DiscovEHR cohort has clinical phenotypes recorded in the GHS EHR over a median of 14 years, with a median of 87 clinical encounters, 658 laboratory tests, and seven procedures captured per participant. Demographics and patient counts for a selection of cardiometabolic, respiratory, neurocognitive, and oncology diseases are described in Table 1.

We sequenced the protein-coding regions of 18,852 genes in 50,726 DiscovEHR participants. Sequence coverage was sufficient to provide at least 20× haploid read depth at >85% of targeted bases in 96% of samples (about 80× mean haploid read depth of targeted bases; fig. S1). We also performed genome-wide array genotyping using the Omni Express exome platform. Per person, we identified a median of 21,409 single-nucleotide variants (SNVs) and 1031 indel variants in protein-coding regions of the genome (fig. S2); a median of 887 variants in each individual was novel. Among all study participants, we identified a total of 4,028,206 unique SNVs and 224,100 unique indels (Table 2), of which 98% occurred at an alternative allele frequency of less than 1%. This abundance of rare variants is consistent with recent accelerated population growth in European-American populations, which comprise an overwhelming majority of the DiscovEHR cohort (fig. S4), and weak purifying selection (2, 24, 25).

Although our ascertainment protocols did not specifically target families, we expected close familial relationships in this stable regional health care population. We therefore examined the extent of these relationships inferred from whole-exome sequence data using Pedigree Reconstruction and Identification of the Maximally Unrelated Set (PRIMUS) (26). Forty-eight percent of sequenced participants had one or more first- or second-degree relatives in the data set (fig. S5), comprising more than 6000 pedigrees with a median pedigree size of two sequenced individuals. The fraction of the autosomal genome existing in runs of homozygosity, which arise from shared parental ancestry, was consistent with previous estimates for European and European-derived populations (27) (fig. S6). Collectively, these findings indicate substantial familial substructure in the DiscovEHR cohort, with modest rates of autozygosity similar to other outbred European populations (27).

Distribution and clinical impact of predicted loss-of-function variants

Each individual had a median of 21 rare variants predicted to result in a loss of gene function (predicted LoFs or pLoFs) and several hundred more common pLoFs (table S1); an average of 43% of these pLoF variants were frameshift indels, and the remainder were SNVs. We found 176,365 pLoF variants among all study participants, of which 114,340 (65%) are predicted to cause loss of function of all RefSeq (28) transcripts. Functionally

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Table 1. Demographics and clinical characteristics of adult (≥18 years old) DiscovEHR study population. Unless otherwise noted, values are expressed as median (interquartile range). Diseases are defined by International Classification of Disease, Ninth Edition (ICD-9) diagnosis codes.		
Basic demographics	GHS active patients	DiscovEHR sequenced patients
N	1,219,522	50,726
Female, n (%)	651,248 (53)	30,028 (59)
Age, years	48 (29–65)	61 (48–73)
Body mass index, kg/m ²	27 (23–32)	30 (28–33)
Years of EHR data	5 (1–11)	14 (11–17)
Medication orders per patient	18 (5–65)	129 (37–221)
Laboratory results per patient	116 (38–368)	658 (197–1,119)
Race		
American Indian or Alaska Native, n (%)	1,344 (0.1)	51 (0.1)
Asian, n (%)	9,625 (0.8)	129 (0.3)
Black or African American, n (%)	41,861 (3)	547 (1)
Native Hawaiian or other Pacific Islander, n (%)	3,306 (0.3)	41 (0.08)
Other, n (%)	6,347 (0.5)	3 (0.01)
Unknown, n (%)	23,319 (2)	63 (0.1)
White, n (%)	1,133,720 (93)	49,892 (98)
Ethnicity		
Hispanic or Latino, n (%)	35,516 (3)	549 (1)
Not Hispanic or Latino, n (%)	863,354 (71)	48,477 (96)
Unknown, n (%)	320,652 (26)	1,700 (3)
Cardiometabolic phenotypes		
Coronary artery disease, n (%)	64,043 (5)	12,298 (24)
Type 2 diabetes, n (%)	87,185 (7)	11,474 (23)
Heart failure, n (%)	43,807 (4)	5,596 (11)
Bariatric surgery, n (%)	6,258 (0.5)	3,112 (6)
Respiratory and immunological phenotypes		
COPD, n (%)	55,278 (5)	6,181 (12)
Asthma, n (%)	83,901 (7)	7,363 (15)
Rheumatoid arthritis, n (%)	10,964 (1)	1,586 (3)
Ulcerative colitis, n (%)	4,708 (0.4)	553 (1)
Neurodegenerative phenotypes		
Alzheimer's disease, n (%)	6,605 (0.5)	233 (0.5)
Parkinson's disease, n (%)	6,513 (0.5)	555 (1)
Multiple sclerosis, n (%)	4,349 (0.4)	487 (1)
Myasthenia gravis, n (%)	735 (0.06)	90 (0.2)
Oncology phenotypes		
Breast cancer, n (%)	15,752 (1)	1,362 (3)
Prostate cancer, n (%)	11,268 (1)	1,349 (3)
Lung cancer, n (%)	7,398 (0.6)	550 (1)
Colorectal cancer, n (%)	7,272 (0.6)	616 (1)

Table 2. Sequence variants identified using whole-exome sequencing of 50,726 DiscovEHR participants.		
Variant type	All variants	Allele frequency ≤1%
SNVs	4,028,206	3,947,488
Insertion/deletion variants	224,100	218,785
Predicted loss-of-function variants	176,365	175,393
Nonsynonymous variants	2,025,800	2,002,912
Total	4,252,306	4,166,273

deleterious SNVs and indels exhibited an allele frequency spectrum skewed toward rarity as compared with other SNVs (Fig. 1, A and B): 55.1% of pLoF SNVs and 58.3% of pLoF indels were singletons compared with 46.5% of all SNVs and 49.9% of all indels. The proportion of pLoF variant sites with a derived allele frequency of <0.1% [fraction of rare variants (FRV), 98.5%] was greater than that of missense variants (97.2%) and synonymous variants (95.4%). This is consistent with previous reports of higher FRV for more functionally impactful variants (13, 29). We observed a higher abundance of pLoF variants in the terminal portion of transcripts, suggesting greater tolerance to pLoF variants that result in near-full-length proteins, as previously described (8) (fig. S7). Examination of the ratio of observed to possible predicted premature stop mutations (12) (Fig. 1, C and D) revealed lower tolerance to pLoF variation in essential genes, cancer-associated genes, and genes associated with autosomal dominant human diseases than in genes associated with autosomal recessive disease genes, drug targets, and olfactory receptors. These results suggest that pLoF variants are under stronger purifying selection compared to variants of other functional classes and that functional context can influence tolerance of genes to pLoF variation.

We estimated the accrual of sequence variants by functional class as sample size grows (Fig. 1, E and F). At the current sample size, rare pLoF variants were observed in 92.4% (17,414) of targeted genes in at least one individual; 15,525 genes (82.4%) harbored rare pLoFs in at least one individual that are predicted to cause loss of function of all protein-coding transcripts of that gene. Homozygous pLoF variants were found in at least one individual in 7.0% of targeted genes (1313 genes), and 868 genes (4.6% of targeted genes) harbored rare pLoFs that affected all transcripts of that gene (Table 3). Of these genes harboring homozygous pLoFs, 654 (49.8%) have not been observed to harbor homozygous pLoFs in other surveys (table S2). This collection of partial and complete human gene knockouts provides opportunities for phenotypic association discovery for highly deleterious gene variants.

To assess the clinical impact of partial or complete loss of gene function in humans, we performed gene-based burden tests of association in mixed linear models with 80 EHR-documented laboratory traits, adjusting for sex, age, and genetic estimates of ancestry and using an experiment-wide significance criterion of 3.3×10^{-8} [0.05/(18,852 genes $\times$ 80 traits); tables S3 and S4]. The most statistically significant association for genes harboring at least one homozygous pLoF was between pLoFs in *CSF2RB*, encoding colony-stimulating factor receptor 2 β common subunit, and basophils [β = -0.58 standard deviations (SDs) per allele, P = 8.6×10^{-15}]. These associations are consistent with loss of function of *CSF2RB*, the common β chain of the high-affinity receptors for the basophil- and eosinophil-inducing cytokines interleukin-3 (IL-3), IL-5, and the cytokine and myeloid differentiation factor granulocyte-macrophage colony-stimulating

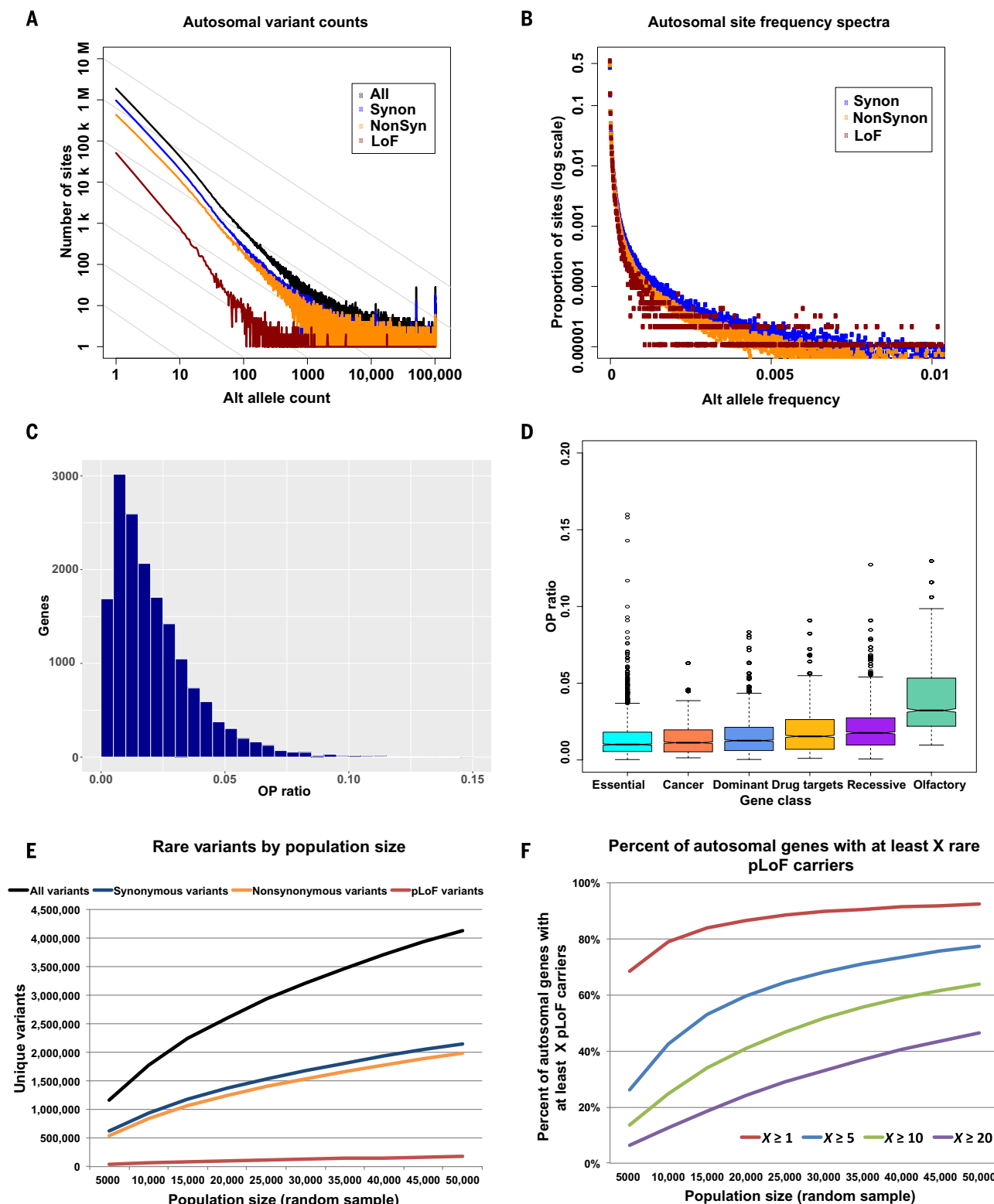


Fig. 1. Frequency and distribution of functional variants in 50,726 exome sequences. (A) Relationship between alternate allele count and SNV site number by functional class. (B) SNV site frequency spectra by functional class, demonstrating enrichment for rare alleles among more functionally deleterious variants. (C) Distribution of observed/predicted (OP) ratio of premature stop variants in 50,726 exome sequences. (D) Distribution of the OP ratio of premature stop variants in 50,726 exome sequences by gene class: essential, mouse essential genes (73); cancer, cancer predisposition genes (74); domi-

nant, autosomal dominant disease genes curated from Online Mendelian Inheritance in Man (OMIM) (75, 76); drug targets, genes encoding targets of 202 drugs (77); recessive, autosomal recessive disease genes curated from OMIM; olfactory, olfactory receptor genes. (E) Accrual of rare (alternate allele frequency < 1%) variants by functional class. (F) Percentage of autosomal genes with multiple predicted loss-of-function (pLoF) carriers as a function of sample size, estimated by randomly sampling the 50,726 sequenced individuals in increments of 5000, creating 10 samples for each increment.

Table 3. Number of genes affected by predicted loss-of-function variants with an allele frequency of ≤1% in 50,726 DiscovEHR participants.

Number of participants	Number of genes affected (%)		
	All, N (%)	Heterozygotes, n (%)	Homozygotes, n (%)
≥1	17,414 (92)	17,409 (92)	1313 (7)
≥5	14,608 (77)	14,598 (77)	312 (2)
≥10	12,105 (64)	12,093 (64)	161 (1)
≥20	8,815 (47)	8,803 (47)	81 (0.4)

factor (30). Homozygous truncating variants in *CSF2RB* have been identified in individuals with pulmonary surfactant metabolism dysfunction-5 [MIM# 614370], characterized by pulmonary alveolar proteinosis (PAP) in which there is excessive deposition of extracellular basophilic globular material (31, 32). Review of structured clinical data (problem list entries, encounter diagnosis codes, procedures, medications, and family history) from EHRs of a single pLoF homozygote revealed diagnoses of chronic cough and pulmonary mycobacterial infection, which are common manifestations of PAP (33, 34).

Among genes harboring only heterozygous pLoF variants, we observed experiment-wide significant associations with calcium (*CASR*), thyrotropin (*TG* and *TSHR*), hepatic transaminases (*GPT* and *GOTT*), alkaline phosphatase (*GPLDI* and *ASGRI*), bilirubin (*SLC01B3*), and hematological traits (*TET2*, *GMPR*, *TUBB1*, *ASXLI*, *HBB*, and *EGLNI*), in addition to lipid associations highlighted below. The association between pLoF variants in *EGLNI*, encoding egl-9 family hypoxia-inducible factor 1, and hemoglobin ($\beta = 1.24$ SD, $P = 5.4 \times 10^{-10}$) and hematocrit ($\beta = 1.28$ SD, $P = 1.4 \times 10^{-10}$) was driven by a single, previously unidentified frameshift variant, p.Pro165fs, carried by 22 individuals. Seven of these p.Pro165fs heterozygotes cosegregated with erythrocytosis in pedigrees reconstructed from exome sequence data, consistent with the gene's described role in familial erythrocytosis 3 [MIM# 609820] (35, 36) (fig. S8). These examples illustrate the utility of pairing pLoF variant discovery with comprehensive EHR-derived clinical phenotypes to understand gene function in humans.

Exome-wide association discovery for serum lipids

To further explore phenotypic associations with coding variants in the DiscovEHR population, we performed an exome-wide association study for fasting lipid levels [total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides], which are risk factors for ischemic vascular diseases, such as coronary artery disease and stroke. A total of 39,087 participants of European-American ancestry from the DiscovEHR cohort had recorded fasting lipid levels with a median of 6 measurements per individual. We performed a mixed linear model analysis of the association between rank inverse normal transformed residuals of

median EHR-documented lipid levels (adjusted for lipid-lowering medication use, sex, age, and genetic estimates of ancestry) and 160,341 biallelic single variants with a minor allele frequency of >0.1%. Using an exome-wide significance criterion of $P < 1 \times 10^{-7}$, we identified 58 SNVs or indel variants (30 nonsynonymous or predicted RNA splice disrupting) in 17 loci with exome-wide significant associations with total cholesterol, 64 variants (29 nonsynonymous or splice) in 21 loci with exome-wide significant associations with HDL-C, 59 variants (27 nonsynonymous or splice) in 14 loci with exome-wide significant associations with LDL-C, and 66 variants (30 nonsynonymous or splice) in 14 loci with exome-wide significant associations with triglycerides (figs. S9 to S12 and tables S5 to S8).

Consistent with other reports (14, 37, 38), we observed an inverse association between allele frequency and effect size (Fig. 2A) and were able to find, for example, three independent exome-wide significant associations with lipid levels for rare single variants: rs138326449-A in *APOC3* (IVS2+1G>A, allele frequency of 0.2%), which was associated with lower triglyceride levels ($\beta = -1.27$ SD, $P = 1.4 \times 10^{-52}$) and higher HDL-C levels ($\beta = 0.85$ SD, $P = 4.3 \times 10^{-24}$); rs12713843-T in *APOB* (p.Arg1128His, allele frequency of 0.5%) associated with lower LDL-C levels ($\beta = -0.33$ SD, $P = 9.4 \times 10^{-10}$) and lower total cholesterol levels ($\beta = -0.30$ SD, $P = 2.0 \times 10^{-8}$); and rs72658867-A (allele frequency of 0.1%), an intronic variant in *LDLR* associated with lower LDL-C levels ($\beta = -0.30$ SD, 1.4×10^{-14}) and lower total cholesterol levels ($\beta = -0.27$ SD, $P = 7.1 \times 10^{-12}$), which corroborates a recent report of a similar association with LDL-C levels for this rare variant (14).

We also performed gene-based burden tests of association in mixed linear models for pLoFs and predicted deleterious nonsynonymous variants. This analysis led to the identification of previously unidentified rare alleles in known lipid-associated gene loci (table S9), which, in aggregate, achieved exome-wide levels of significance for gene-based burden testing ($P < 1 \times 10^{-6}$). Among these was an association between heterozygous pLoF or deleterious missense variants in 994 individuals in *CD36*, a broadly expressed membrane glycoprotein that serves as a receptor for various ligands, including oxidized lipoproteins and fatty acids (39), and HDL-C levels ($\beta = 0.20$ SD, $P = 3.4 \times 10^{-7}$). A role in HDL-C uptake in the liver has been proposed by studies of *Cd36*-deficient mice (40), and

common variation at the *CD36* locus has been associated with HDL-C levels in African Americans (41, 42). Our results provide further evidence of a role for *CD36* in the modulation of HDL-C levels in individuals of European ancestry.

An association between *G6PC* and lipid levels was newly implicated by the burden test: 288 heterozygous carriers of 36 pLoF or predicted deleterious missense variants in *G6PC* had significantly higher triglyceride levels ($\beta = 0.35$ SD, $P = 5.2 \times 10^{-7}$; lipid values by variant in Fig. 2B). *G6PC* encodes glucose-6 phosphatase catalytic subunits. Homozygous and compound heterozygous mutations in *G6PC* are associated with glycogen storage disease type Ia [MIM# 232200], a canonically autosomal recessive disease that is characterized by lipid and glycogen accumulation in the liver and kidneys accompanied by hypoglycemia, lactic acidosis, hyperuricemia, and hyperlipidemia (43). Gene-based burden tests of association between *G6PC* and 709 ICD-9 (*International Classification of Disease, Ninth Edition*)-derived clinical disease phenotype groups with a case frequency of >1% (table S9) identified associations with gout [odds ratio (OR), 2.02; 95% confidence interval (CI), 1.39 to 2.93; $P = 0.0002$] and gouty arthropathy (OR, 2.58; 95% CI, 1.54 to 4.33; $P = 0.0003$), in addition to tension headache (OR, 2.99; 95% CI, 1.67 to 5.33; $P = 0.0002$). Association testing with median uric acid levels extracted from EHR data from 11,540 DiscovEHR participants, of whom 23 carried a pLoF or deleterious missense variant in *G6PC*, identified nominally significantly higher uric acid levels ($\beta = 0.27$ SD, $P = 0.01$). Thus, heterozygotes for protein-disrupting variants in *G6PC* appear to manifest an intermediate phenotype characterized by moderate levels of hypertriglyceridemia and increased risk for gout and gouty arthropathy.

Loss-of-function variants in lipid drug target genes and lipid levels

Human genetic variants that inactivate genes encoding drug targets may mimic the action of therapeutic antagonism of these targets, thereby providing an experiment of nature that may be used to infer the clinical effects of drug antagonists of that target. We evaluated associations between pLoF variants, aggregated by genes, in nine therapeutic targets of drugs for lipid modification and lipid levels extracted from the EHR (Fig. 3 and table S11). Of these drug target genes, six of nine harbored pLoF variants that were at least nominally associated with changes in lipid levels, recapitulating clinical effects of the therapeutic agent [15 total nominal ($P < 0.05$) associations among 36 tests]. Among all gene-based burden association tests between pLoFs and lipid levels ($n = 75,408$ tests), we observed 4335 nominal associations. Thus, under the null expectation of no association between pLoFs in lipid drug genes and lipid levels, we expected 2 nominally significant associations among 36 association tests, demonstrating ~7.5-fold enrichment for nominal associations with lipid drug target genes ($P = 4.3 \times 10^{-10}$ by exact binomial test).

Among currently approved therapeutics, these observations confirm associations between rare pLoF variants in *NPC1L1* ($n = 137$ heterozygotes), which encodes the target of ezetimibe, and *PCSK9* ($n = 49$ heterozygotes), which encodes the target of alirocumab and evolocumab and reduced LDL-C levels (16–18, 44), mirroring the clinical effects of therapeutic antagonism of these genes; pLoFs in *PCSK9* were associated with the greatest reduction in LDL-C levels. We also observed highly statistically significant associations between heterozygous pLoF variants in *APOB* and reduced LDL-C and triglyceride levels among 58 pLoF carriers, recapitulating the effect of therapeutic antagonism with mipomersen, an antisense oligonucleotide to apo-B100 (45, 46). Homozygous or compound heterozygous truncating mutations in *APOB* have been implicated in familial hypobetalipoproteinemia-1 [MIM# 615558], characterized by profound depression of apolipoprotein B (apoB)-containing lipoproteins, including LDL-C and triglyceride-rich lipoproteins, and hepatic triglyceride accumulation due to decreased secretion (47). Association testing with median alanine and aspartate aminotransferase levels revealed higher levels of both hepatic transaminases ($\beta = 0.12$ SD, $P = 0.08$ and $\beta = 0.27$, $P = 0.02$ for alanine and aspartate aminotransferase levels, respectively) in pLoF variant carriers compared to noncarriers. Consistent with autosomal codominant transmission of certain clinical features of hypobetalipo-

proteinemia, *APOB* pLoF heterozygotes in the DiscovEHR cohort manifest an intermediate phenotype characterized by moderate depression of LDL-C and triglyceride levels and elevated transaminase levels, suggesting hepatocyte injury. These associations mirror the clinical effects of mipomersen, including elevation in hepatic transaminases in some treated patients (45, 46). In contrast, 29 individuals from the DiscovEHR cohort who were heterozygous for predicted loss-of-function mutations in *MTTP* did not have lipid levels that were significantly different from noncarriers, suggesting that *MTTP*-associated abetalipoproteinemia [MIM# 200100] segregates exclusively as a recessive trait in our study population.

We observed an association between pLoF variants in *CETP*, encoding the target of anacetrapib (currently in phase 3 clinical trials), and higher HDL-C ($\beta = 0.82$ SD, $P = 2.9 \times 10^{-6}$). Two of three genes encoding targets of therapeutic agents currently in phase 2 clinical trials for lipid modification (*APOC3* and *ANGPTL3*) harbored pLoFs that were associated with lipid profiles, recapitulating therapeutic effects. Nine heterozygotes for pLoF variants in *ACLY*, a target gene of the *ACLY*-antagonist bempedoic acid in phase 2 clinical trials for lipid lowering, had a trend toward lower LDL-C values ($\beta = -0.67$ SD, $P = 0.07$). These findings suggest that coding variation coupled to EHR-derived clinical phenotypes can recover associations that validate drug targets,

anticipate on-target adverse effects, and potentially reveal previously unidentified targets for therapeutic development.

Prevalence of clinically returnable genetic findings in 50,726 exomes

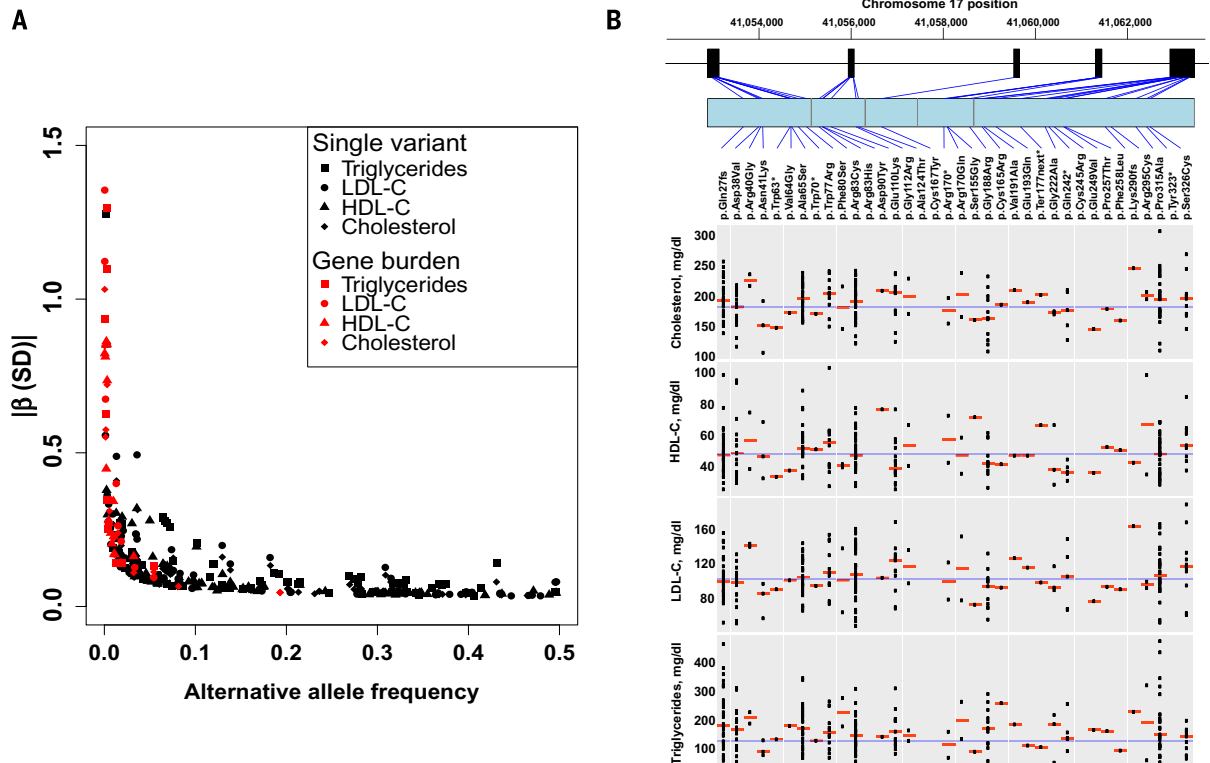
Exome sequence data were analyzed to identify potentially pathogenic variants, according to the ClinVar “pathogenic” classification (48), in a subset of 76 genes [Geisinger-76 (G76)] that, when altered, lead to clinically actionable findings for 27 medical conditions (table S12). The G76 includes the 56 genes and 25 conditions identified in the American College of Medical Genetics and Genomics (ACMG) recommendations for identification and reporting of clinically actionable genetic findings (49), as well as an additional 17 genes associated with the same 25 conditions and 3 genes associated with 2 additional conditions. All genes and conditions were chosen on the basis of being associated with clinically actionable and highly penetrant monogenic disease. In addition to identifying variants documented to be pathogenic in ClinVar, we identified pLoF variants that were potentially pathogenic (“expected pathogenic”), as recommended by the ACMG guidelines (49).

In aggregate, 6653 individuals (13% of sequenced participants) harbored one or more such pathogenic or expected pathogenic variants in the G76 gene list: 5435 individuals with at least one variant

Fig. 2. Exome-wide association discovery for fasting lipid levels. (A)

Relationship between allele frequency and effect size for single-variant and gene burden tests of association with lipid levels. Effect size is given as the absolute value of β , in SD units. Only single-variant and gene-based burden associations meeting exome-wide significance criteria (1×10^{-7} and 1×10^{-6} for single-variant and gene-based burden tests of association) are displayed. (B)

Lipid values by variant for predicted loss of function and predicted deleterious missense variants in *G6PC*. Red lines indicate the median values for variant carriers. Each dot represents a trait value for a single carrier of the variants specified above each box. The blue line indicates the median value for all sequenced individuals not carrying a predicted loss of function or predicted deleterious missense variant in *G6PC*. To convert values for cholesterol to millimoles per liter, multiply by 0.0259. To convert the values for triglycerides to millimoles per liter, multiply by 0.0113.



in these genes with a pathogenic classification in ClinVar and 1218 additional participants with predicted pathogenic pLoF variants. A pilot set of 1415 sequence files then underwent clinical curation, applying clinical laboratory standards (50) for potential return to clinical care. Within this pilot set, 641 variants in the G76 were reviewed: 49 were considered either “pathogenic” ($n = 32$, 5.0%) or “likely pathogenic” ($n = 17$, 2.7%), and the remaining 592 (93.3%) were considered either variants of uncertain significance, likely benign, benign, or false positives. Of the variants classified in the pilot sample as pathogenic or likely pathogenic by a Clinical Laboratory Improvement Amendments–certified molecular diagnostic laboratory, 43 reportable variants (all either heterozygous for autosomal dominant conditions or hemizygous for X-linked conditions) occurred in 49 of the 1415 participants’ samples; 3 individuals carried 2 such variants associated with two distinct diseases (table S13). Therefore, 3.5% of the DiscovEHR cohort participants are estimated to have a variant from the G76 that meets or exceeds current clinical standards for asserting pathogenicity, namely, >90% certainty of the variant being disease-causing (50). We investigated the expressivity of these variants by reviewing structured EHR-derived clinical phenotype data for variant carriers. Collectively, 32 of 49 individuals (65%) had clinical features in the EHR that were consistent with the associated disease: 7 (14%) individuals had a diagnosis code entry consistent with a formal diagnosis of the associated disease, and

an additional 26 (53%) individuals had diagnosis codes or family history entries consistent with clinical features of the associated disease (table S13). In a companion publication, we describe the prevalence, familial segregation, and clinical impact of variants associated with familial hypercholesterolemia, highlighting the opportunity for early clinical intervention afforded by genetic diagnosis of this condition (51). These results demonstrate the potential for genomics-guided clinical care in a large unselected clinical population, establish an expectation for the burden of actionable genetic findings, and support the need for expert clinical review and adjudication of assertions of pathogenicity for potentially pathogenic variants, including those cataloged in mutation databases.

Conclusions

The findings reported here demonstrate the value of large-scale sequencing in a clinical population from an integrated health system and add to the knowledge base regarding human genetic variation provided by other large-population genetic surveys (1, 2, 52). Megaphenic variants and known pathogenic alleles of clinical relevance have been observed in the protein-coding regions of the genome (3, 49, 53). As purifying selection ensures that these variants are kept at very low frequencies, very large populations are needed to identify rare variants with large effects on clinical traits (54). The discovery of pLoF and other functionally impactful variants in nearly all genes, coupled

with structured clinical data in EHRs, provides a rich resource to study the phenotypic effects of partial and complete gene knockouts in humans, as well as other forms of genetic variation. These data may inform discovery of previously unidentified biological mechanisms and therapeutic targets and facilitate interrogation of gene-centric hypotheses around specific phenotypic associations of potential clinical value. Although many of these variants are rare individually, in aggregate, they are not uncommon, and their identification and the biological insights gleaned are relevant to our understanding and treatment of both common and rare diseases.

The DiscovEHR collaboration represents a powerful platform for human genetics research and allows us to fill gaps in our knowledge regarding the role of genetic variation in determining health and disease and the implementation of genomic information in routine medical care. Large-scale sequencing initiatives in integrated health care systems, such as the DiscovEHR collaboration, hold great promise for genetic discoveries to advance precision medicine.

Materials and methods
The DiscovEHR collaboration cohort

The DiscovEHR collaboration study cohort is derived from individuals who consented to participate in Geisinger’s MyCode Community Health Initiative. As described in detail elsewhere (23), the MyCode initiative leverages the resources of Geisinger as an integrated health care delivery

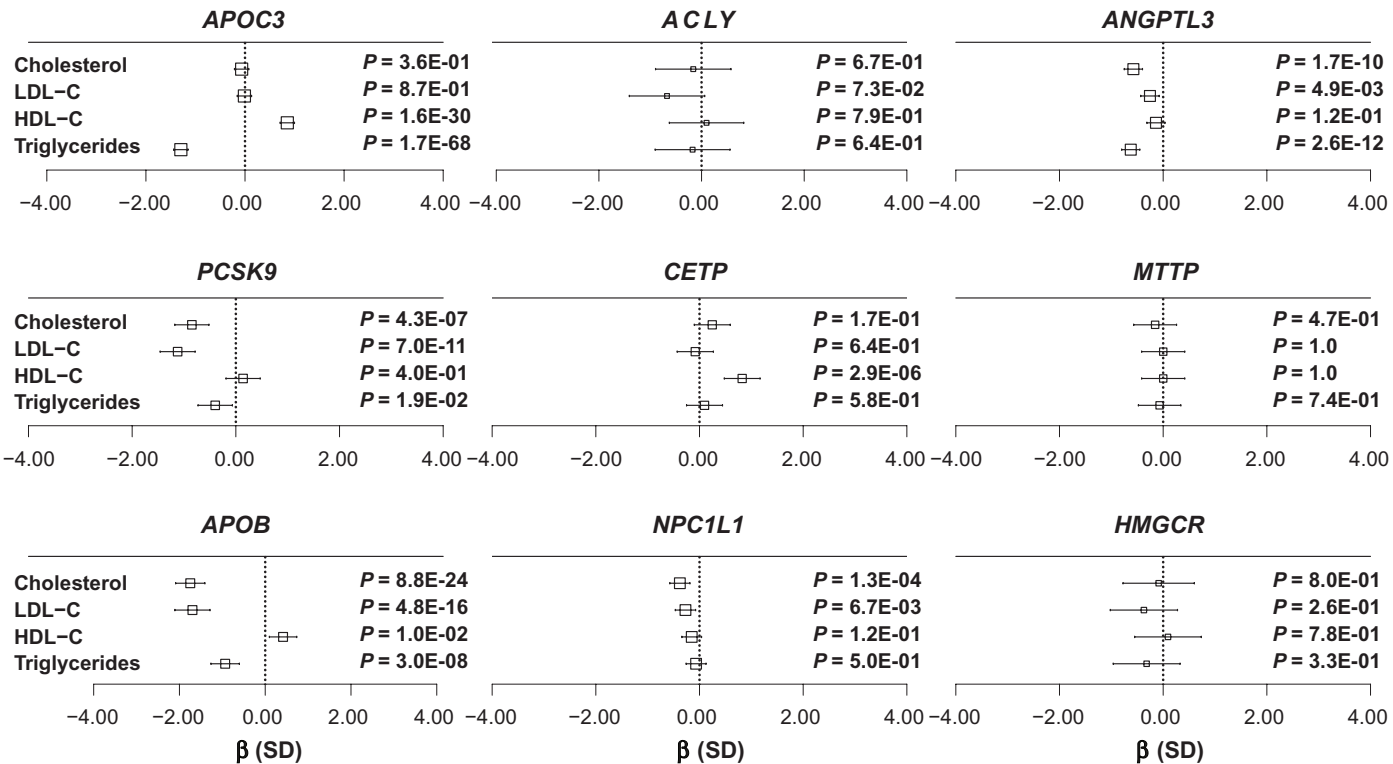


Fig. 3. Associations between predicted loss-of-function variants in lipid drug target genes and lipid levels. Boxes correspond to effect size, in SD units; whiskers denote 95% CIs for β . The size of the box is proportional to the logarithm (base 10) of predicted loss-of-function carriers.

system for implementation of precision medicine. All MyCode participants are enrolled through an opt-in informed consent process; participants agree to provide samples for broad, future research use, including genetic analysis, and to linking of samples and data to information in their GHS EHR; participants agree to be contacted in the future to receive additional information or to invite them to participate in additional research projects; and participants agree to receive clinically actionable findings, the sharing of that information with their clinical providers, and placement of the information in their EHR. Geisinger uses a “data-broker” system to protect confidentiality. Participants are informed that their samples or information might be shared with external research collaborators in a manner that would protect their privacy and confidentiality of their information. To date, more than 125,000 GHS patients have consented to enroll in MyCode, with an 86% enrollment rate for those invited to participate. Enrollment is ongoing.

The DiscovEHR cohort that provides the basis for the findings reported here includes the first 50,726 adult MyCode participants from whom DNA samples were obtained. This includes 6,672 individuals recruited from the Geisinger cardiac catheterization laboratory and 2,785 individuals from the bariatric surgery clinic, with the remaining 41,269 individuals representing an otherwise unselected GHS outpatient population.

Sample preparation and sequencing

Sample preparation and whole-exome sequencing were performed as described (55). In brief, sample quantity was determined by fluorescence (Life Technologies) and quality assessed by running 100ng of sample on a 2% pre-cast agarose gel (Life Technologies). The DNA samples were normalized and one aliquot was sent for genotyping (Illumina, Human OmniExpress Exome Beadchip) and another sheared to an average fragment length of 150 base pairs using focused acoustic energy (Covaris LE220). The sheared genomic DNA was prepared for exome capture with a custom reagent kit from Kapa Biosystems using a fully-automated approach developed at the Regeneron Genetics Center. A unique 6 base pair barcode was added to each DNA fragment during library preparation to facilitate multiplexed exome capture and sequencing. Equal amounts of sample were pooled prior to exome capture with NimbleGen probes (SeqCap VCRome). Captured fragments were bound to streptavidin-conjugated beads and non-specific DNA fragments removed by a series of stringent washes according to the manufacturer’s recommended protocol (Roche NimbleGen). The captured DNA was PCR amplified and quantified by qRT-PCR (Kapa Biosystems). The multiplexed samples were sequenced using 75 bp paired-end sequencing on an Illumina v4 HiSeq 2500 to a coverage depth sufficient to provide greater than 20x haploid read depth of over 85% of targeted bases in 96% of samples (approximately 80x mean haploid read depth of targeted bases).

Sequence alignment, variant identification, and genotype assignment

Upon completion of sequencing, raw sequence data from each Illumina HiSeq 2500 run was gathered in local buffer storage and uploaded to the DNAnexus platform (56) for automated analysis. After upload was complete, analysis began with the conversion of BCL files to FASTQ-formatted reads and assigned, via specific barcodes, to samples using the CASAVA software package (Illumina Inc., San Diego, CA). Sample-specific FASTQ files, representing all the reads generated for that sample, were then aligned to the GRCh37.p13 genome reference with BWA-mem (57). The resultant binary alignment file (BAM) for each sample contained the mapped reads’ genomic coordinates, quality information, and the degree to which a particular read differed from the reference at its mapped location. Aligned reads in the BAM file were then evaluated to identify and flag duplicate reads with the Picard MarkDuplicates tool (<http://picard.sourceforge.net>), producing an alignment file (duplicatesMarked.BAM) with all potential duplicate reads marked for exclusion in later analyses.

Variant calls were produced using the Genome Analysis Toolkit (GATK) (58). GATK was used to conduct local realignment of the aligned, duplicate-marked reads of each sample around putative indels. GATK’s HaplotypeCaller was then used to process the INDEL-realigned, duplicate-marked reads to identify all exonic positions at which a sample varied from the genome reference in the genomic VCF format (GVCF). Genotyping was accomplished using GATK’s GenotypeGVCFs on each sample and a training set of 50 randomly selected samples, previously run at the Regeneron Genetics Center (RGC), outputting a single-sample VCF file identifying both SNVs and indels as compared to the reference. Additionally, each VCF file carried the zygosity of each variant, read counts of both reference & alternate alleles, genotype quality representing the confidence of the genotype call, the overall quality of the variant call at that position, and the QualityByDepth for every variant site.

Variant Quality Score Recalibration (VQSR), from GATK, was employed to evaluate the overall quality score of a sample’s variants using training datasets (e.g., 1000 Genomes) to assess and recalculate each variant’s score, increasing specificity. Metric statistics were captured for each sample to evaluate capture, alignment, and variant calling using Picard, bcftools (<http://samtools.github.io/bcftools>), and FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc).

Following completion of cohort sequencing, samples showing disagreement between genetically-determined and reported sex (n=143); low quality DNA sequence data indicated by high rates of heterozygosity or low sequence data coverage (less than 75% of targeted bases achieving 20X coverage) (n=181); or genetically-identified sample duplicates (n=222) were excluded (n=494 unique samples excluded). Following these exclusions, 51,298 exome sequences were available for downstream analysis, and we report here findings

from exome sequences corresponding to 50,726 individuals who were 18 years of age or older at the time of initial consent. These samples were used to compile a project-level VCF (PVCF) for downstream analysis. The PVCF was created in a multi-step process using GATK’s GenotypeGVCFs to jointly call genotypes across blocks of 200 samples, recalibrated with VQSR and aggregated into a single, cohort-wide PVCF using GATK’s CombineVCFs. Care was taken to carry all homozygous reference, heterozygous, homozygous alternate, and no-call genotypes into the project-level VCF. For the purposes of downstream analyses, samples with QD < 5.0 and DP < 10 from the single sample pipeline had genotype information converted to ‘No-Call’, and variants falling more than 20 bp outside of the target region were excluded.

Sequence annotation and identification of functional variants

Sequence variants were annotated with snpEff (59) using the Ensembl75 gene definitions to determine their functional impact on transcripts and genes. In order to reduce the number of false-positive pLOF calls related to inaccurate transcript definitions, a “whiteList” set of 56,507 protein-coding transcripts that have an annotated start and stop codon (corresponding to 19,729 genes) were selected as a reference for functional annotations. These transcripts were also flagged to allow for downstream filtering for the following features: a) small introns (<15bp), b) small exons (<15bp), c) non-canonical splice sites (non-“GT/AG” splice sites).

The snpEff predictions that correspond to the “whiteList” filtered transcripts are then collapsed into a single most-deleterious functional impact prediction (i.e., the Regeneron Effect Prediction) by selecting the most deleterious functional effect class for each gene according to the hierarchy in table S1. Predicted loss of function mutations were defined as SNVs resulting in a premature stop codon, loss of a start or stop codon, or disruption of canonical splice dinucleotides; open-reading-frame shifting indels, or indels disrupting a start or stop codon, or indels disrupting of canonical splice dinucleotides (table S14). Predicted loss of function variants that correspond to the ancestral allele or that occur in the last 5% of all affected transcripts were excluded.

Principal components and ancestry estimation

We performed principal component (PC) analysis in PLINK2 (60) using the subset of overlapping variant sites (n=6,331) from DiscovEHR whole-exome sequence and the 1000 genomes Omni chip platform. This analysis was further restricted to common (MAF>5%) autosomal variant sites with high genotyping rate (>90%) in both Hardy-Weinberg ($p > 1 \times 10^{-6}$) and linkage equilibrium which did not map to the MHC region (n sites after filters = 3,974). Initial calculations were based on 1000 genomes v2 (61) samples and DiscovEHR individuals were projected onto these PC’s.

To identify a subset of European individuals within DiscovEHR, we constructed a linear model

trained on PCs estimates from 1000 genomes known ancestry groups (EUR, ASN, AFR) using the first three PCs. Thresholds for each model (EUR=0.9, AFR=0.7, ASN=0.8) were applied to determine the best continental ancestry match for each DiscovEHR individual; we designated samples not meeting any of these thresholds as “Admixed”. Within the DiscovEHR European population, we calculated a new set of PCs for the maximum unrelated set of individuals using similar variant filtering criteria. Related individuals within DiscovEHR were subsequently projected onto these PCs. These European-only PCs calculated from unrelated DiscovEHR individuals were used in phenotype association analyses.

Relationship estimation

Pairwise identity-by-descent (IBD) estimates were calculated using PLINK2 (60) and were used to reconstruct pedigrees with PRIMUS (26). First, we used common variants (MAF >10% in Hardy-Weinberg-Equilibrium (p-val > 0.000001) to calculate IBD proportions all pairs of samples, excluding individuals with >10% missing variant calls (–mind 0.1) and abnormally low inbreeding-coefficient (–0.15) calculated with the –het option in PLINK. We then removed samples with >100 relatives with $\pi_{\text{hat}} > 0.1875$ if the proportion of relatives with $\pi_{\text{hat}} > 0.1875$ was less than 40% of the sample's total relationships determined by a π_{hat} of 0.05, and removed all samples with >300 relatives. The remaining samples were grouped into family networks. Two individuals are in the same network if they were predicted to be second-degree relatives or closer. We then ran the IBD pipeline implemented in PRIMUS to calculate accurate IBD estimates among samples within each family network. This approach allowed for better-matched reference allele frequencies to calculate relationships within each family network.

Analysis of runs of homozygosity

Analysis of runs of homozygosity (ROH), which arise from shared parental ancestry in an individual's pedigree, is a powerful approach to estimate the extent of ancient kinship and recent parental relationship within a population. Typically, offspring of cousins have long ROH, commonly over 10 Mb. By contrast, almost all Europeans have ROH of ~2 Mb in length, reflecting shared ancestry from hundreds to thousands of years ago. By focusing on ROH of different lengths, it is therefore possible to infer aspects of demographic history at different time depths in the past (52). We used FROH measures to compare and contrast DiscovEHR to populations from 1000 genomes. These measures are genomic equivalents of the pedigree inbreeding coefficient, but do not suffer from problems of pedigree reconstruction. By varying the lengths of ROH that are counted, they may be tuned to assess parental kinship at different points in the past. We used FROH5, the fraction of the autosomal genome existing in ROH over 5Mb in length, reflective of a parental relationship in the last four to six generations, as our metric of autozygosity.

For a subset of DiscovEHR individuals for which Omni HumanOmniExpressExome-8v1-2 genotype data were available (N=34,246), genotypes were merged with 1092 individuals from 1000 genomes phase I. ROH were identified using PLINK2 (60). We performed LD-based SNP pruning in windows of 50kb with a step size of 5 variants and r-squared threshold of 0.2. On the pruned subset of variants (n=114,514), we applied the following parameters for calculating ROH: 5 MB window size; a minimum of 100 homozygous SNPs per ROH; a minimum of 50 SNPs per ROH window; one heterozygous and five missing calls per window; a maximum between-variant distance within a run of homozygosity of no more than 1Mb. ROH were identified separately for DiscovEHR individuals and for each 1000 genomes population.

We assessed three features of ROH: (i) number of homozygous segments (average and range, calculated across individuals within a population), (ii) summed segment length (average and range, calculated across individuals within a population) and (iii) FROH5, a genomic measure of individual autozygosity, defined as the proportion of the autosomal genome in ROH in runs of 5 Mb or greater in length(52).

For DiscovEHR individuals, we note a mean FROH5 of 0.0006. For CEU individuals, we note a mean FROH5 of 0.0008. This is consistent with previous estimates for European and European-derived populations, where HapMap CEU individuals also had a mean FROH5 of 0.0008 and English individuals had a mean FROH5 of 0.0001 (27). We conclude that as a population as a whole, DiscovEHR individual have level of genomic autozygosity that is lower than CEU and only slightly higher than individuals from England.

Phenotype definitions

ICD-9 based diagnosis codes were collapsed to hierarchical clinical disease groups and corresponding controls using a modified version of the groupings proposed by Denny et al (62, 63). ICD-9 based diagnoses required one or more of the following: a problem list entry of the diagnosis code or an encounter diagnosis code entered for two separate clinical encounters on separate calendar days. Median values for selected serially measured laboratory with serial outpatient measures (table S3) were calculated for all individuals with two or more measurements in the EHR following removal of likely spurious values that were > 3 standard deviations from the intra-individual median value. For the purposes of exome-wide association analysis of serum lipid levels, total cholesterol and LDL-C were adjusted for lipid-altering medication use by dividing by 0.8 and 0.7, respectively, to estimate pre-treatment lipid values based on the average reduction in LDL-C and total cholesterol for the average statin dose (64). HDL-C and triglyceride values were not adjusted for lipid-altering medication use. We then calculated trait residuals for all laboratory traits after adjustment for age, age², sex, and the first ten principal components of ancestry, and rank-inverse-normal transformed these residuals prior to association analysis.

Association analysis for serum lipid levels and other laboratory values

In single-marker exome-wide association analysis of lipid levels, we analyzed all bi-allelic variants with missingness rates < 1%, Hardy-Weinberg equilibrium p values > 1.0x10⁻⁶, and minor allele frequency > 0.1%. Genotypes were coded according to an additive genotypic model (0 for homozygous reference, 1 for heterozygous, and 2 for homozygous alternative). To account for population structure from ancestry and relatedness, we used mixed linear models of association to test for associations between single variants and lipid trait residuals, fitting a genetic relatedness matrix (constructed from 39,858 non-MHC markers in approximate linkage equilibrium with minor allele frequency > 0.1%) as a random-effects covariate.

We next used the same statistical testing framework to identify gene-based associations between variants, aggregated over the gene (65) with the traits enumerated above. We used three variant sets for this gene-based allele aggregation:

1. Predicted loss of function mutations.
2. Predicted loss of function mutations and non-synonymous variants that were predicted deleterious by consensus of 5/5 algorithms [SIFT (66), LRT (67), MutationTaster (68), PolyPhen2 HumDiv, PolyPhen2 HumVar (69)].
3. Predicted loss of function mutations and rare (alternative allele frequency < 1%) non-synonymous variants that were predicted deleterious by at least 1/5 algorithms.

Alleles were coded 0,1,2 for non-carriers, heterozygotes for at least one variant site but not homozygous at any variant site, and homozygotes for at least one variant site in each variant set, respectively. Exome-wide quantile-quantile plots and genomic control lambda values for single-marker and gene-based burden tests are provided in figs. S7 to S10. The tests all appeared to be well-calibrated. GCTA v1.2.4 (70) and R version 3.2.1 (R Project for Statistical Computing) were used for all statistical analyses.

Exploratory gene-based association analyses with 709 disease phenotypes with case frequency greater than 1% were performed using logistic regression (to provide point estimates of odds ratios), adjusted for age, age², sex, and the first ten principal components of ancestry, and BOLT-LMM (7) (to provide p-values), adjusted for age, age², sex, and genetic relatedness. Wald 95% confidence intervals were estimated for odds-ratios using standard error estimates back-calculated from p-values from the linear mixed models of association in BOLT-LMM. Alleles were coded as above. For association testing for pLoFs, aggregated by gene, and 80 clinical laboratory trait values, residualized as described above, we used BOLT-LMM.

Identification of expected and known pathogenic variants in genes associated with medically actionable diseases

To estimate the burden of potentially returnable genetic findings in all 50,726 sequenced participants, we extracted all the coding variants

identified in the ACMG 56 recommended gene list (49) and additional GHS 20 genes for returnable secondary findings. We cross-referenced these variants with the ClinVar dataset [updated December 2015] restricting to those with a Pathogenic classification and a minor allele frequency of less than 1% in the DiscovEHR population. We also cross-referenced the variants with the Human Gene Mutation Database [HGMD 2015.4] restricting to DM-Disease causing mutations of High-confidence variants only with a MAF <1%. We compiled the list of returnable variants according to the published guidelines for the genes where Expected Pathogenic (EP), comprising non-reported putative loss-of-function (pLoF), and/or Known Pathogenic (KP) variants are recommended for clinically actionable return of results.

For a subset of sequenced samples, we applied an orthogonal workflow for variant discovery, adjudication of asserted pathogenicity, and development of clinical reports. We used a custom pipeline to annotate and prioritize clinically important variants. This pipeline incorporates standard annotations, including variant effect, protein functional predictions, conservation, and allele frequencies, along with public and private databases of variants. Variants within the genes of interest were filtered to extract all known alleles of reported clinical relevance and rare, novel and likely disruptive variants (e.g. frame-shift, nonsense, splicing, etc). After potentially important variants were identified, they were subjected to manual assessment.

Manual assessments were performed as previously described (72), and were consistent with the process developed through a national effort to establish standardized clinical classification criteria (50). Briefly, variants were assessed using a comprehensive evidence review, which included published case-control, genetic and functional data as well as population frequency and predictive assessment data. Classifications were subsequently assigned according to a 5-tier system (pathogenic, likely pathogenic, uncertain significance, likely benign, benign). For autosomal dominant disorders, reported variants were limited to those classified as likely pathogenic or pathogenic for the diseases of interest. For autosomal recessive and X-linked disorders, (i.e. *MUTYH*-associated polyposis), likely pathogenic or pathogenic variants were only reported when present in a homozygous, compound heterozygous, or hemizygous state. All reported variants were confirmed via Sanger sequencing.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6319/aaf6814/suppl/DC1
Figs. S1 to S12
Tables S1 to S14

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RESEARCH ARTICLE SUMMARY

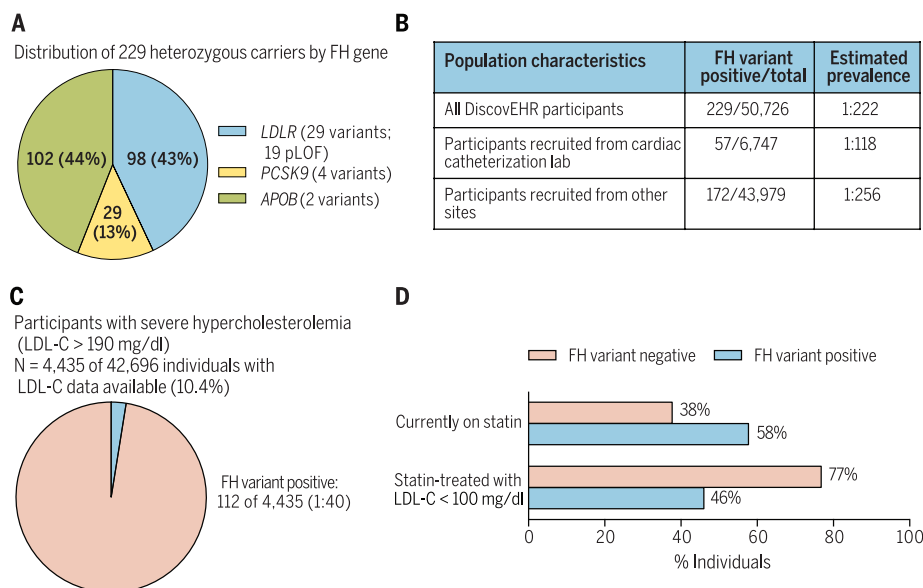
HUMAN GENETICS

Genetic identification of familial hypercholesterolemia within a single U.S. health care system

Noura S. Abul-Husn, Kandanurugu Manickam, Laney K. Jones, Eric A. Wright, Dustin N. Hartzel, Claudia Gonzaga-Jauregui, Colm O'Dushlaine, Joseph B. Leader, H. Lester Kirchner, D'Andra M. Lindbuchler, Marci L. Barr, Monica A. Giovanni, Marylyn D. Ritchie, John D. Overton, Jeffrey G. Reid, Raghu P. R. Metpally, Amr H. Wardeh, Ingrid B. Borecki, George D. Yancopoulos, Aris Baras, Alan R. Shuldiner, Omri Gottesman, David H. Ledbetter, David J. Carey, Frederick E. Dewey, Michael F. Murray*

INTRODUCTION: Familial hypercholesterolemia (FH) is a public health genomics priority but remains underdiagnosed and undertreated despite widespread cholesterol screening. This represents a missed opportunity to prevent FH-associated cardiovascular morbidity and mortality. Pathogenic variants in three genes (*LDLR*, *APOB*, and *PCSK9*) account for the majority of FH cases. We assessed the prevalence and clinical impact of FH-associated genomic variants in 50,726 individuals from the MyCode Community Health Initiative at Geisinger Health System who underwent exome sequencing as part of the DiscovEHR human genetics collaboration with the Regeneron Genetics Center.

RATIONALE: Genetic testing for FH is uncommon in clinical practice in the United States, and the prevalence of FH variants in U.S. populations has not been well established. We sought to evaluate FH prevalence in a large integrated U.S. health care system using genomic sequencing and electronic health record (EHR) data. We determined the impact of FH variants on low-density lipoprotein cholesterol (LDL-C) levels and coronary artery disease (CAD) risk. We assessed the likelihood of FH variant carriers achieving a presequencing EHR-based FH diagnosis according to established clinical diagnostic criteria. Finally, we examined the rates of statin medication use and outcomes in FH variant carriers.



Prevalence and clinical impact of FH variants in a large U.S. clinical care cohort. (A) Distribution of 229 heterozygous carriers of an FH variant in the DiscovEHR cohort by FH gene. (B) Prevalence of an FH variant in the DiscovEHR cohort and according to recruitment site. (C) Prevalence of an FH variant among individuals with severe hypercholesterolemia (LDL-C $\geq$ 190 mg/dl). (D) Statin treatment rates and outcomes in FH variant carriers and noncarriers.

RESULTS: Thirty-five known and predicted pathogenic variants in *LDLR*, *APOB*, and *PCSK9* were identified in 229 individuals. The estimated FH prevalence was 1:256 in unselected participants and 1:118 in participants ascertained via the cardiac catheterization laboratory. FH variants were found in only 2.5% of individuals with severe hypercholesterolemia (maximum EHR-documented LDL-C $\geq$ 190 mg/dl) in the cohort, and a maximum LDL-C of $\geq$ 190 mg/dl was absent in 45% of FH variant carriers. Overall, FH variant carriers had 69 ± 3 mg/dl greater maximum LDL-C than sequenced noncarriers ($P = 1.8 \times 10^{-20}$) and had significantly increased odds of general and premature CAD

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[odds ratio (OR), 2.6 ($P = 4.3 \times 10^{-11}$) and 3.7 ($P = 5.5 \times 10^{-14}$), respectively]. The increased odds of general and premature CAD were most pronounced in carriers of *LDLR* predicted loss-of-function variants [OR, 5.5 ($P = 7.7 \times 10^{-13}$) and 10.3 ($P = 9.8 \times 10^{-19}$), respectively]. Fourteen FH variant carriers were deceased; chart review revealed that none of these individuals had a clinical diagnosis of FH. Before genetic testing, only 15% of FH variant carriers had an ICD-10 (International Classification of Diseases, 10th revision) diagnosis code for pure hypercholesterolemia or had been seen in a lipid clinic, suggesting that few had been previously diagnosed with FH. Retrospectively applying Dutch Lipid Clinic Network diagnostic criteria to EHR data, we found presequencing criteria supporting a probable or definite clinical diagnosis of FH in 24% of FH variant carriers, highlighting the limitations of using existing clinical criteria for EHR-based screening in the absence of genetic testing. Active statin use was identified in 58% and high-intensity statin use in 37% of FH variant carriers. Only 46% of carriers currently on statin therapy had a most recent LDL-C level below 100 mg/dl compared to 77% of noncarriers.

CONCLUSION: In summary, we show that large-scale genomic screening in patients with longitudinal EHR data has the ability to detect FH, uncover and characterize novel pathogenic variants, determine disease prevalence, and enhance overall knowledge of clinical impact and outcomes. The 1:256 prevalence of FH variants in this predominantly European-American cohort is in line with prevalence estimates from recent work in European cohorts. Our findings highlight the undertreatment of FH variant carriers and demonstrate a potential clinical benefit for large-scale sequencing initiatives in service of precision medicine. ■

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RESEARCH ARTICLE

HUMAN GENETICS

Genetic identification of familial hypercholesterolemia within a single U.S. health care system

Noura S. Abul-Husn,¹ Kandamurugu Manickam,² Laney K. Jones,² Eric A. Wright,² Dustin N. Hartzel,² Claudia Gonzaga-Jauregui,¹ Colm O'Dushlaine,¹ Joseph B. Leader,² H. Lester Kirchner,² D'Andra M. Lindbuchler,² Marci L. Barr,² Monica A. Giovanni,² Marylyn D. Ritchie,² John D. Overton,¹ Jeffrey G. Reid,¹ Raghu P. R. Metpally,² Amr H. Wardeh,² Ingrid B. Borecki,¹ George D. Yancopoulos,¹ Aris Baras,¹ Alan R. Shuldiner,¹ Omri Gottesman,¹ David H. Ledbetter,² David J. Carey,² Frederick E. Dewey,¹ Michael F. Murray^{2*}

Familial hypercholesterolemia (FH) remains underdiagnosed despite widespread cholesterol screening. Exome sequencing and electronic health record (EHR) data of 50,726 individuals were used to assess the prevalence and clinical impact of FH-associated genomic variants in the Geisinger Health System. The estimated FH prevalence was 1:256 in unselected participants and 1:118 in participants ascertained via the cardiac catheterization laboratory. FH variant carriers had significantly increased risk of coronary artery disease. Only 24% of carriers met EHR-based presequencing criteria for probable or definite FH diagnosis. Active statin use was identified in 58% of carriers; 46% of statin-treated carriers had a low-density lipoprotein cholesterol level below 100 mg/dl. Thus, we find that genomic screening can prompt the diagnosis of FH patients, most of whom are receiving inadequate lipid-lowering therapy.

Familial hypercholesterolemia (FH) is one of three genomic conditions designated by the Centers for Disease Control and Prevention as having potential for a significant positive impact on public health through improved diagnosis and treatment (1, 2). FH is characterized by substantial, lifelong elevation of low-density lipoprotein cholesterol (LDL-C) and a markedly increased risk of premature cardiovascular disease (3, 4). Known genetic causes of FH include inactivating variants in the gene encoding the LDL receptor (*LDLR*), protein-disrupting variants in apolipoprotein B (*APOB*), and activating variants in proprotein convertase subtilisin/kexin type 9 (*PCSK9*). Although estimated at a worldwide prevalence of 1:500 (5–7), recent studies in some European countries have revealed that FH could affect ~1:250 individuals (8, 9), with higher prevalence observed in certain founder populations (10, 11). The prevalence of FH in U.S. populations is not well established. Notably, despite widespread cholesterol screening, only a small fraction of FH cases are appropriately diagnosed and treated (4, 12, 13). This represents a missed opportunity to prevent FH-associated cardiovascular morbidity and mortality.

A diagnosis of FH can be made with a validated set of criteria, such as those established by

the Dutch Lipid Clinic Network (DLCN), Simon Broome, or Make Early Diagnosis to Prevent Early Death (MEDPED) (14–17). These diagnostic tools estimate the likelihood of FH on the basis of clinical features and, in the case of DLCN and Simon Broome criteria, also include identification of functional variants in the *LDLR*, *APOB*, or *PCSK9* genes. However, genetic testing for these variants is uncommon in clinical practice in the United States. We thus sought to understand the prevalence and clinical impact of FH variants in a clinical cohort by analyzing genomic sequence and electronic health record (EHR) data from 50,726 individuals from the Geisinger Health System, an integrated health care system with provider services in Pennsylvania and New Jersey.

Exome sequencing of 50,726 individuals reveals a high genotypic prevalence of FH

This study included 50,726 consented adult participants from the MyCode Community Health Initiative of Geisinger Health System (18) who underwent exome sequencing as part of the DiscovEHR human genetics study (see table S1 for demographics and clinical characteristics of the study population) (19). Participants were 59.2% female, with a median age of 61 years, and predominantly Caucasian (98.4%). LDL-C values were available in the EHR for 42,696 (84.2%) of sequenced participants; of these, 4435 (10.4%) had severe hypercholesterolemia, defined as maximum EHR-documented LDL-C $\geq$ 190 mg/dl (20).

Statin use was documented at any point in the EHR in 27,402 (54.0%) of sequenced participants.

The exome sequence data were analyzed for known pathogenic variants in *LDLR*, *PCSK9*, and *APOB* and for predicted protein-truncating, loss-of-function (LoF) variants in *LDLR* (table S2), including exonic copy number variants identified via CLAMMS (Copy number estimation using Lattice-Aligned Mixture Models) (21). By positional intersection with the clinical genetics database ClinVar (22), we found 19 missense variants in *LDLR*, *PCSK9*, and *APOB* designated as “pathogenic” for FH. We identified 21 additional predicted LoF variants in *LDLR*, including 9 frameshift, 8 splice donor or acceptor, and 4 nonsense variants. We also identified a predicted pathogenic exon 13 to 17 tandem duplication in *LDLR*; this was the only copy number variant identified in *LDLR*. Upon manual review, six variants were removed to produce a more stringent set of variants (tables S1 and S2); these included two missense variants with conflicting evidence in ClinVar, two missense variants that were located at the same amino acid residue as a pathogenic variant but were not in ClinVar themselves, and two that were annotated as splice variants in an alternative transcript of *LDLR*, for which there is no evidence of protein expression. Thus, 35 known (that is, ClinVar-documented) and predicted (that is, protein-truncating LoF) pathogenic FH variants (29 *LDLR*, 4 *PCSK9*, and 2 *APOB* variants) were used for our analyses.

Among the 50,726 sequenced participants, we identified 229 heterozygous carriers of 1 of the 35 FH variants, corresponding to a total carrier frequency of 1:222 participants (Table 1). There were no cases of homozygous or compound heterozygous FH. Given that this prevalence estimate is based on a sampling of individuals within a single health care delivery system, it may be an overestimation of population frequency due to ascertainment bias. The MyCode cohort included 6747 participants recruited from the cardiac catheterization laboratory; the estimated prevalence of an FH variant among these participants was 1:118, and the prevalence in other participants was 1:256 (Table 1). Overall, 98 (42.8%) individuals were found with *LDLR* variants, 102 (44.5%) with *APOB* variants, and 29 (12.7%) with *PCSK9* variants (table S2). A recent study by Khera *et al.* (23) identified more individuals with variants in *LDLR* (86%) and relatively fewer *APOB* (13%) and *PCSK9* (0.6%); this discrepancy is likely due to the inclusion of different populations in each study, with theirs being 46% South Asian and 7% black.

We identified 30 first- or second-degree relationships among FH variant carriers using their exome data (24), including two pedigrees containing five sequenced noncarriers and eight carriers of the *APOB* p.Arg3527Gln variant, where segregation of high LDL-C levels with carrier status can be observed (fig. S1). The three variants with the largest number of related carriers accounted for more than half of FH cases in the study: *APOB* p.Arg3558Cys (46 carriers, 8 related), *APOB* p.Arg3527Gln (56 carriers, 10 related), and

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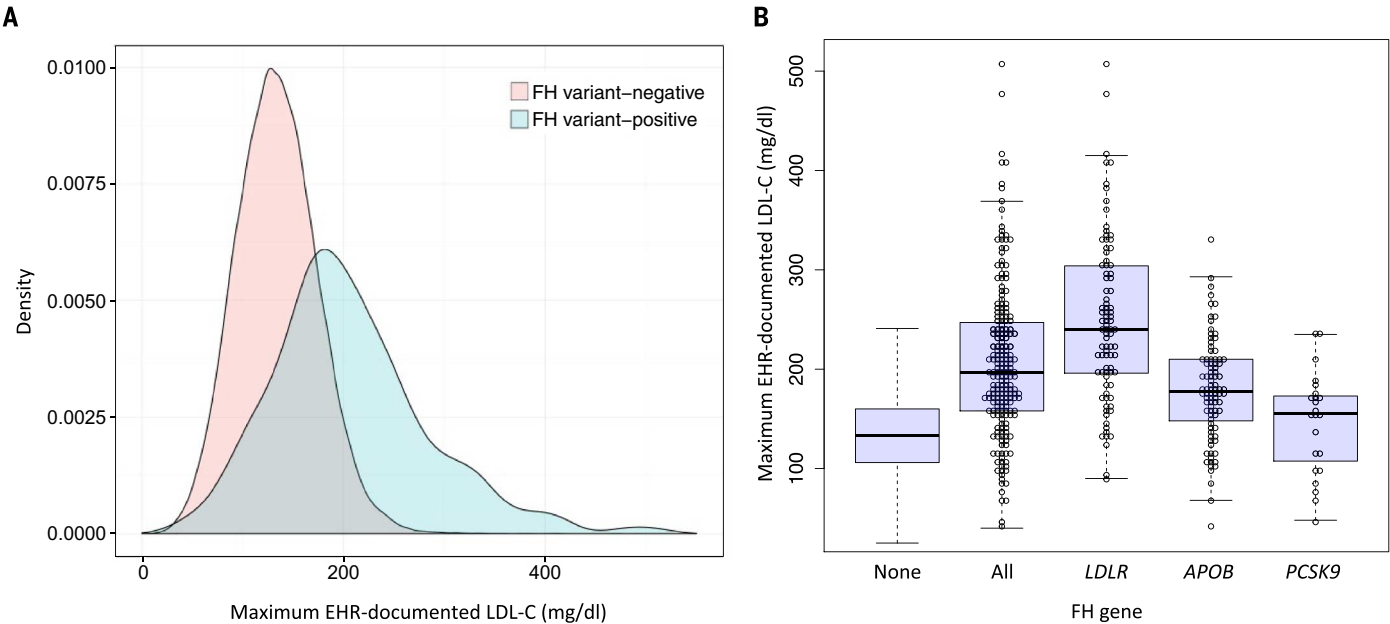


Fig. 1. FH variants are associated with increased LDL-C levels. (A) Density of maximum EHR-documented LDL-C levels in heterozygous carriers of any FH variant (FH variant-positive, $n = 204$) and in sequenced noncarriers (FH variant-negative, $n = 42,442$) with LDL-C data available in the EHR. LDL-C levels approximated a normal distribution (Anderson-Darling normality test; $P < 2.2 \times 10^{-16}$ in FH variant-negative and $P = 6.1 \times 10^{-5}$ in FH variant-positive). FH variant carriers had 69 ± 3 mg/dl greater LDL-C than non-carriers in a mixed linear model analysis adjusting for age, age², sex, and the first five principal components of ancestry ($P < 1.8 \times 10^{-20}$). (B) Maximum EHR-documented LDL-C levels in sequenced noncarriers ($n = 42,442$) and

in carriers according to FH gene ($n = 88, 92$, and 24 for *LDLR*, *APOB*, and *PCSK9*, respectively). Open circles indicate individual LDL-C values for FH variant carriers. Median LDL-C level (in mg/dl) and IQR are shown in the box plots; these were 133.0 (106.0 to 160.0) in noncarriers, 240.3 (196.5 to 303.5) in *LDLR*, 178 (148.0 to 210.0) in *APOB*, and 155.0 (107.5 to 173.0) in *PCSK9* variant carriers. Maximum LDL-C was higher in carriers of variants in *LDLR* compared to *APOB* or *PCSK9* (one-way ANOVA with post hoc Tukey test; $P = 1.5 \times 10^{-10}$ and $P = 1.3 \times 10^{-9}$, respectively). There was no significant difference in maximum LDL-C between carriers of *APOB* and *PCSK9* variants ($P = 0.09$).

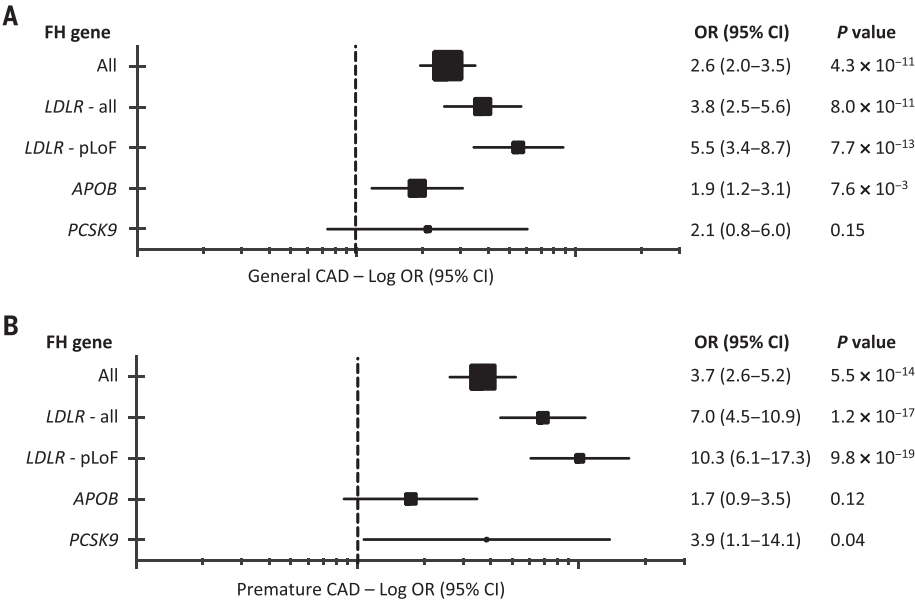


Fig. 2. FH variants are associated with increased risk of CAD. CAD cases and controls were defined with ICD-9 (International Classification of Diseases, Ninth Revision) diagnosis codes and cardiac catheterization report data extracted from EHRs. ORs for general CAD (A) and premature CAD (B) (defined as males ≤ 55 years and females ≤ 65 years) were calculated by logistic regression with adjustment for age, sex, and principal components of ancestry (see table S3). pLoF, predicted LoF (defined as variants leading to a premature stop codon, or loss of a start or stop codon; disrupting canonical splice acceptor or donor dinucleotides; or frameshifting leading to the formation of a premature stop codon); *LDLR* - all, all known and predicted pathogenic variants identified in *LDLR*; *LDLR* - pLoF, predicted LoF variants identified in *LDLR*.

the *LDLR* exon 13 to 17 duplication (29 carriers, 10 related). This underscores the likelihood of encountering close family members with FH even in an unselected clinical population within a regional health care system and the opportunity for family-based screening and clinical management. In a subset of the sequenced cohort in which only one individual in every first- and second-degree relationship was retained, the overall prevalence of an FH variant remained unchanged at 1:224 (Table 1).

FH variant carriers have higher LDL-C levels than noncarriers and are at increased cardiovascular risk

LDL-C levels were available in the EHR for 204 of 229 FH variant-positive and for 42,442 FH variant-negative individuals. We examined maximum EHR-documented LDL-C levels in the sequenced cohort to approximate the untreated or pretreated state. Among FH variant-positive and FH variant-negative individuals, maximum LDL-C levels approximated a normal distribution (Fig. 1A). Carriers of any FH variant had 69 ± 3 mg/dl greater maximum LDL-C than sequenced noncarriers in a mixed linear model analysis ($P = 1.8 \times 10^{-20}$). Maximum LDL-C values were significantly higher in carriers of *LDLR* variants [median, 240.3 mg/dl; interquartile range (IQR), 196.5 to 303.5] compared to carriers of *APOB* [median, 178.0 mg/dl; IQR, 148.0 to 210.0; one-way

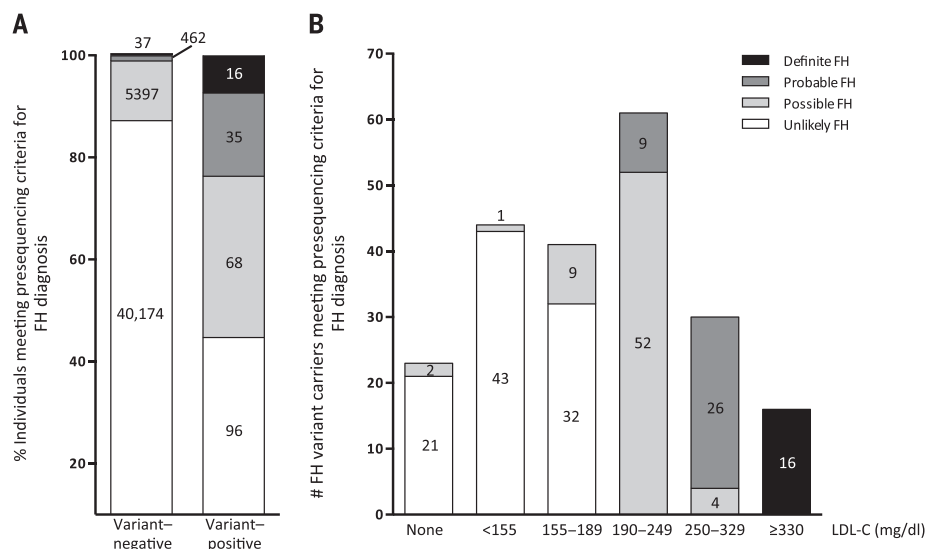


Fig. 3. Presequencing likelihood of FH diagnosis with DLCN criteria. Criteria for diagnosis of FH (unlikely, possible, probable, or definite) were based exclusively on extracted EHR data. **(A)** Percentage of participants meeting clinical criteria for FH diagnosis among living noncarriers (variant-negative; $n = 46,070$) and carriers (variant-positive; $n = 215$) of an FH variant. **(B)** Number of living variant carriers ($n = 215$) that would meet presequencing criteria for FH diagnosis per stratum of maximum EHR-documented LDL-C range (in mg/dl).

analysis of variance (ANOVA) with post hoc Tukey test, $P = 1.5 \times 10^{-10}$] or *PCSK9* (median, 155.0 mg/dl; IQR, 107.5 to 173.0; $P = 1.3 \times 10^{-9}$) variants (Fig. 1B). Despite overall increased LDL-C in FH carriers, maximum LDL-C levels for each identified FH variant were widely variable, ranging from a median of 90 to 479 mg/dl (table S2).

The frequency of known and predicted pathogenic FH variants was evaluated across categories of maximum EHR-documented LDL-C levels (Table 1 and table S1). A significantly higher proportion of FH variant carriers (112 of 204 or 54.9%) had severe hypercholesterolemia (maximum LDL-C ≥ 190 mg/dl) compared to noncarriers (4309 of 42,442 or 10.2%; χ^2 test, $P < 0.0001$; table S1). The prevalence of an FH variant increased significantly across increasing thresholds of maximum LDL-C levels (Table 1; Cochran-Armitage test for trend, $P < 0.0001$). Of the 4435 sequenced participants with LDL-C ≥ 190 mg/dl, only 112 harbored an FH variant (2.5% or 1:40). Of the 53 with maximum LDL-C ≥ 330 mg/dl, 17 had an FH variant (32.1% or 1:3).

We evaluated the association of known and predicted pathogenic FH variants with coronary artery disease (CAD) as defined by electronic phenotyping of CAD cases and controls through the EHR (25), using linear mixed models to account for population structure due to ancestry and relatedness (Fig. 2 and table S3). Individuals with an FH variant had increased odds of CAD [odds ratio (OR), 2.6; 95% confidence interval (CI), 2.0 to 3.5; $P = 4.3 \times 10^{-11}$] compared to noncarriers. The increased risk of CAD was greatest in carriers of *LDLR* predicted LoF variants (OR, 5.5; 95% CI, 3.4 to 8.7; $P = 7.7 \times 10^{-13}$) and smallest in carriers of *APOB* variants (OR, 1.9;

95% CI, 1.2 to 3.1; $P = 7.6 \times 10^{-3}$). We identified 4150 individuals with premature CAD (defined as having CAD ≤ 55 years in males and ≤ 65 years in females) in the cohort. Of these, 53 individuals harbored an FH variant, indicating a prevalence of genotypically defined FH among individuals with premature CAD of 1:78 (1.3%). The odds of premature CAD were significantly increased among FH variant carriers (OR, 3.7; 95% CI, 2.6 to 5.2; $P = 5.5 \times 10^{-14}$). As was observed with general CAD, the increased risk of premature CAD was greatest in carriers of *LDLR* predicted LoF variants (OR, 10.3; 95% CI, 6.1 to 17.3; $P = 9.8 \times 10^{-19}$) and smallest in carriers of *APOB* variants (OR, 1.7; 95% CI, 0.9 to 3.5; $P = 0.12$). Notably, 14 of the 229 FH variant carriers were deceased (mean age of death, 76.1 years; range, 58 to 91 years). Chart review revealed that the cause of death was cardiovascular-related in half of these individuals and that 13 had evidence of potential FH-related comorbid disease (table S4). None of the deceased FH variant carriers carried a clinical diagnosis of FH.

EHR-based FH diagnosis is challenging without knowledge of an FH variant

We evaluated whether any of the 229 FH variant carriers might have been previously diagnosed with FH by mining the EHRs for a diagnosis code of “pure hypercholesterolemia” or for any encounter at Geisinger’s specialized Lipid Clinic (table S1); these were present in the EHRs of only 35 (15.3%) carriers.

We retrospectively applied the DLCN criteria for FH diagnosis to EHR data available from living individuals in the sequenced MyCode cohort (table S5). These criteria allow for a diagnosis of FH on the basis of clinical and family history of

elevated LDL-C, premature cardiovascular disease, physical stigmata of hypercholesterolemia, and, when known, molecular identification of FH variants (16). Among living variant-negative individuals ($n = 46,070$), there were 37 (0.1%) “definite,” 462 (1.0%) “probable,” and 5397 (11.7%) “possible” FH diagnoses solely on the basis of EHR data (Fig. 3A). Among living variant-positive individuals ($n = 215$), there were 16 (7.4%) definite, 35 (16.3%) probable, and 68 (31.6%) possible FH diagnoses (Fig. 3A and table S6). Individuals meeting probable or definite criteria for FH diagnosis (23.7% of carriers) were clustered among those with a maximum LDL-C level of ≥ 190 mg/dl (Fig. 3B). There were 96 (44.7%) variant carriers who would have been judged unlikely to have a diagnosis of FH; there were no significant differences in age or other characteristics between these individuals and those in the combined possible/probable/definite FH categories (table S6). When the same DLCN criteria were applied with the inclusion of identified FH variants, 188 (87.4%) individuals met criteria for definite and 27 (12.6%) met criteria for probable FH diagnosis. Application of MEDPED criteria (17) to EHRs produced similar results, with 53 (24.7%) living FH variant carriers meeting criteria for clinical FH diagnosis (table S7). Together, these data highlight the limitations of using clinical criteria for EHR-based screening and the opportunity for genomic data to augment the detection of individuals with FH.

Individuals deemed unlikely to have FH (by DLCN criteria) before identification of a pathogenic variant, despite having LDL-C data available in the EHR ($n = 75$), were considered to have nonpenetrant disease. On the basis of these results, we predicted 18 FH variants to have reduced penetrance, which included 13 of 29 *LDLR*, 3 of 4 *PCSK9*, and 2 of 2 *APOB* variants (table S2). This estimate is limited by the incompleteness of EHR data (table S5), the inability to account fully for lipid-lowering treatment effects, the variable number of heterozygous carriers per variant, and familial relationships between some of the carriers within the cohort. For example, closely related carriers may have shared environmental and genetic factors influencing their phenotypes, which could affect estimates of penetrance.

FH variant carriers are undertreated and have LDL-C levels above goal

Overall, 173 (80.5%) of the 215 living FH variant carriers had been prescribed lipid-lowering therapy (mostly statin medications; table S8). We examined the medication reconciliation records from 2015 to 2016 available for 189 of the FH variant carriers. An active prescription for statin medication was identified in 109 (57.7%) FH variant carriers, of whom 70 (37.0%) were prescribed high-intensity statin therapy. Among those not receiving high-intensity statin therapy ($n = 119$), 10 individuals (8.4%) had EHR-documented evidence of statin intolerance. The average lowest LDL-C level among 192 FH variants carriers with these data available was 104.9 mg/dl. Among 106

Table 1. Prevalence of an FH variant in the MyCode cohort. We assessed the prevalence of an FH variant in all sequenced participants, in a subset in which only one individual in each first- and second-degree relationship was retained, according to recruitment site (from the cardiac catheterization laboratory or elsewhere), and across increasing LDL-C thresholds.

Population characteristics	n	FH variant–positive n (%)*	Estimated prevalence
All sequenced participants	50,726	229 (0.5)	1:222
Including only one individual in every first- and second-degree relationship	38,339	171 (0.5)	1:224
Recruitment site†			
Cardiac catheterization laboratory	6,747	57 (0.8)	1:118
Other sites	43,979	172 (0.4)	1:256
Maximum EHR-documented LDL-C (mg/dl)‡			
LDL-C < 155	28,512	48 (0.2)	1:607
LDL-C ≥ 155	14,184	156 (1.1)	1:91
LDL-C ≥ 190	4,435	112 (2.5)	1:40
LDL-C ≥ 250	390	50 (12.8)	1:8
LDL-C ≥ 330	53	17 (32.1)	1:3

*Heterozygous carriers of 1 of the 35 identified FH variants (n = 229 total; n = 204 individuals with LDL-C data available). †n = 50,726 sequenced participants. ‡n = 42,696 sequenced participants with LDL-C data available.

variant carriers with LDL-C levels available within 12 months of the study, the average most recent LDL-C level was 130.3 mg/dl, and only 41 (38.7%) carriers had a most recent LDL-C level below 100 mg/dl, which is the LDL-C target recommended by the National Lipid Association's Expert Panel for adults with FH (26). Subsetting to carriers currently on statin therapy and with recent LDL-C levels available (n = 63), only 29 (46.0%) had a most recent LDL-C level below 100 mg/dl. In comparison, 76.8% of noncarriers currently on statin had a most recent LDL-C below 100 mg/dl (χ^2 test, $P < 0.0001$; table S8).

Conclusion

Underdiagnosis and undertreatment of FH continue to be a concern, and are associated with premature CAD and stroke, indicating that genomic approaches to case identification may be needed as a front-end intervention to improve outcomes (4). Here, exome sequence data linked to longitudinal EHRs identified FH variants at a higher than previously estimated prevalence (6, 7, 10); excluding participants recruited from a cardiac catheterization laboratory, the prevalence was 1:256, in line with more recent estimates of 1:217 in Denmark (8) and 1:319 in the Netherlands (9). Our assessment of FH prevalence in a U.S. health care system using this methodology supports the claim that there is significant underdiagnosis of this condition (4, 12). Whether our estimated prevalence of FH variants in our patient population, largely a stable regional health care population in central Pennsylvania, is generalizable to other U.S. patient populations remains to be determined. We caution that extant familial (and cryptic) relatedness in our study population could result in overestimation of a given FH-causing allele that is rare in the general population but segregating in family members. For example, our study population was enriched for APOB p.Arg3527Gln, which is known to be common in individuals of Amish descent in Lancaster, Pennsylvania (11).

Surprisingly, FH variants explain only 2.5% of severe hypercholesterolemia in the cohort, which challenges the notion that patients with LDL-C levels of more than 190 mg/dl are likely to have genotypically defined FH (27); this is consistent with a recent observation that 1.7% of patients with severe hypercholesterolemia had an FH variant (23). There are several potential explanations for this. Polygenic hypercholesterolemia (28, 29) could account for a proportion of FH variant–negative cases of severe LDL-C elevation, as could common, secondary causes of elevated LDL-C, such as obesity, hypothyroidism, and nephrotic syndrome (20, 30). It is also possible that, by including only established pathogenic variants in LDLR, APOB, and PCSK9, and predicted pathogenic LoF variants in LDLR in our analysis, we excluded additional truly pathogenic FH variants that would explain some cases of severe hypercholesterolemia. Finally, there may be yet-to-be-identified genes that are causative of FH.

We applied a validated set of clinical FH diagnostic criteria to EHR data to estimate the likelihood of achieving a presequencing diagnosis in living FH variant carriers. Only 24% of carriers would have met criteria for probable or definite FH diagnosis before variant identification. This suggests that such diagnostic algorithms may be of limited utility when applied to EHRs in the absence of genetic testing, as others have observed (31), which has direct implications for current efforts seeking to use similar methodologies. Future large-scale EHR screening programs may use natural language processing and machine learning to improve reliability. For example, the FH foundation recently launched the Find FH program, which is developing a machine-learning algorithm to identify individuals with probable FH within EHR data, laboratory results, and claims databases (32).

By surveying EHR records from last year, we found that 58% of FH variant carriers were currently prescribed a statin medication, and only 46% of those on statin treatment had a most

recent LDL-C level under 100 mg/dl, the recommended LDL-C target for adult FH patients (20, 27, 33). These findings are consistent with previous reports and highlight the undertreatment of FH (4, 34). The risk for general and premature CAD was significantly higher in FH variant carriers, with ORs of 2.6 and 3.7, respectively, and this was most pronounced in those with predicted LoF variants in LDLR. A similarly increased risk of CAD was observed by Khera et al. (23), who reported ORs of 3.8 for carriers of any FH variant and 9.5 for predicted LoF variants in LDLR. This underscores the importance of early diagnosis and appropriate treatment of these patients. At present, there are no genotype-based guidelines for the treatment of hyperlipidemia; future studies evaluating the effects of new therapies on cardiovascular disease outcomes may lead to clarification of FH treatment guidelines.

Despite its strengths, this study has limitations. The study population does not necessarily reflect the global community, but rather individuals presenting for clinical care. The average age of sequenced MyCode participants was 61 years; therefore, there may be survival bias in this cohort, with underrepresentation of individuals having severe or homozygous FH (who often do not survive past the second decade if left untreated) (35). The cohort also had a higher percentage of ever-prescribed statin compared to the total ~1.4 million patients in the Geisinger Health System with EHR data (54% versus 13%), although surprisingly few FH variant carriers had an active statin order in the sequenced cohort. As with any real-world study, we were limited by the data collected, which is subject to error in entry and incompleteness, specifically missing data in the EHR (such as physical exam findings and family history). Because major efforts, including the national Precision Medicine Initiative, will also rely heavily on EHR data, this study helps to validate this approach despite the limitations cited. We assumed that the most recent outpatient LDL-C value was reflective

of current prescribed lipid-lowering therapy and that medications were being taken upon physician order and not by independent verification. We did not adjust LDL-C values to account for treatment effect; instead, we elected to analyze maximum EHR-documented LDL-C, because this was most likely to approximate the untreated state in this real-world cohort and because of some degree of uncertainty in the timing of statin initiation relative to EHR-documented LDL-C levels. Other studies have implemented a standard 30% reduction in LDL-C in individuals on statin treatment (23), but note that this does not account for different statin types or doses, or potential variability in response across FH carriers and noncarriers. Finally, we elected to use a conservative definition of pathogenic variants, which did not include missense variants designated as “likely pathogenic” or having in silico predictions of pathogenicity in our analysis; this could have resulted in an underestimate of the true frequency of FH.

This study helps build support for the emerging concept that, in conditions such as FH, genomic sequencing might soon be applied as part of a population screen (36). Given that genomic sequencing has not been routinized in U.S. health care except in research settings such as this, other health care systems would currently require programmatic funding beyond clinical reimbursements to apply a similar approach to their patient population (37). The costs of sequencing, interpretation, and follow-up of secondary and false-positive findings from genomic sequencing would need to be considered. Proof of clinical utility and cost-effectiveness of genomic screening for FH can be built on the Dutch model experience, where screening, diagnosis, and treatment led to more than 3 years of life gained in FH (38). A recent study modeling genetic screening for FH suggested that it would not currently be cost-effective in the United States, although the authors acknowledged several knowledge gaps regarding FH diagnosis, cascade screening, management, and clinical outcomes in the United States, as well as outdated genetic testing cost input data (39). Further analyses taking into account a changed DNA sequencing landscape with rapidly declining costs, and new and more effective treatment options, will be critical in considering implementation of genomic screening for FH and in determining the clinical settings and patient populations that would benefit most from this approach. Genetic identification of FH is just one clinical application of the many possible uses of genomic sequencing, and in and of itself may not justify the cost. Cost-effectiveness and clinical utility models in which genomic sequencing is used as a single test to inform many actionable genetic diseases require further evaluation.

This study targets a particular use of sequencing information, and at this point, we cannot fully model how genomic sequencing as a front-end intervention might alter clinical care by improving detection of FH-conferred risk. Nonetheless, our findings demonstrate a potential clinical benefit for the large-scale sequencing

planned by the national Precision Medicine Initiative (40). An important goal of precision medicine is to accurately identify and treat individuals at increased risk of medically actionable conditions (41); as a highly modifiable genetic condition, FH is an ideal starting point for implementation of a return of results program (42). The clinical impact of systematic genomic screening for FH may be further magnified by cascade screening of family members, identification of novel genetic causes of FH, and protective genetic or other factors to explain nonpenetrance, ultimately leading to more refined cardiovascular risk stratification for patients with elevated LDL-C levels.

Materials and methods

Setting and study participants

Human genetics studies were conducted as part of the DiscovEHR project of the Geisinger Health System (GHS) and the Regeneron Genetics Center (RGC). The study was approved by the GHS Institutional Review Board. The study population consisted of 50,726 consented participants ≥ 18 years from the MyCode® Community Health initiative of GHS, an integrated health services organization in Pennsylvania and New Jersey. A detailed description of the DiscovEHR study population can be found in a companion publication (19).

Sequencing of LDLR, APOB, and PCSK9

Sample preparation and whole exome sequencing were performed at the RGC as previously described (25). In brief, exome capture was performed using NimbleGen probes according to the manufacturer's recommended protocol (Roche NimbleGen). The captured DNA was PCR amplified and quantified by qRT-PCR (Kapa Biosystems). The multiplexed samples were sequenced using 75 bp paired-end sequencing on an Illumina v4 HiSeq 2500 to a coverage depth sufficient to provide greater than 20x haploid read depth of over 85% of targeted bases in 96% of samples (approximately 80x mean haploid read depth of targeted bases). Raw sequence data from each Illumina HiSeq 2500 run were uploaded to the DNAnexus platform (43) for sequence read alignment and variant identification. In brief, raw sequence data were converted from BCL files to sample-specific FASTQ-files, which were aligned to the human reference build GRCh37.p13 with BWA-mem (44). Single nucleotide variants (SNV) and insertion/deletion (indel) sequence variants were identified using the Genome Analysis Toolkit (45, 46). Copy number variants were detected using the CLAMMS algorithm (21).

Sequence data for *LDLR*, *APOB*, and *PCSK9* were extracted from exome sequences generated at the RGC with the use of protocols described in detail in a companion publication (19). Sequence variants were annotated using SnpEff (47) and predicted LoF variants were defined as any of the following: SNVs leading to a premature stop codon, loss of a start codon, or loss of a stop codon; SNVs or indels disrupting canonical splice acceptor or donor dinucleotides; open reading frame shifting indels leading to the formation of a premature stop codon. Sequence variants

annotated by the clinical genetics database ClinVar (22) as “pathogenic” were also identified by positional intersection with exome sequence variant calls. The ClinVar database was accessed in March 2016. We considered LoF variants (previously known and novel) in *LDLR* as “predicted pathogenic” variants, and considered missense variants classified in ClinVar as “pathogenic” in *LDLR*, *APOB*, and *PCSK9* as “known pathogenic” variants. The union of “predicted pathogenic” and “known pathogenic” variants was the set of sequence variations used for all subsequent analyses.

FH diagnostic criteria and clinical characteristics

A clinical diagnosis of FH was established using the DLCN criteria (15, 16). Relevant data were extracted from the EHR and a numerical score was assigned (see table S5). EHR data were unavailable for some of the phenotypic criteria, in particular physical exam findings (tendon xanthomata and arcus cornealis) in patients or relatives. Total scores were calculated without and with the knowledge of a functional variant in *LDLR*, *APOB*, or *PCSK9* genes. A diagnosis of FH was considered definite if the total score was greater than 8, probable if the score was 6–8, possible if the score was 3–5, and unlikely if the score was below 3 points.

We applied MEDPED criteria to EHRs in a similar manner. As MEDPED criteria rely on the knowledge of 1st, 2nd, or 3rd degree relatives of FH (17), which is not captured in the EHR, we expanded the criteria to include knowledge of any family history of hyperlipidemia, and used corresponding MEDPED age-based LDL-C thresholds for individuals with 1st degree relatives with FH (if positive family history of hyperlipidemia) or for general population (if negative family history of hyperlipidemia).

Maximum EHR-documented LDL-C values and lowest EHR-documented LDL-C values were extracted from EHRs. We extracted most recent EHR-documented LDL-C values from levels recorded in the EHR within 12 months of the study. Only outpatient LDL-C measurements are reported. Evidence of ever-prescribed lipid-lowering therapy was also extracted from the EHR. Current lipid-lowering therapy was defined as the last prescribed lipid-lowering agent documented in the EHR in patients with a medical reconciliation performed in 2015 or 2016. “High-intensity statins” were defined as high doses of specific statin medications: simvastatin 80 mg, atorvastatin 40 or 80 mg, and rosuvastatin 20 or 40 mg daily (20). Moderate-intensity statins were defined as moderate doses of specific statin medications: atorvastatin 10 or 20 mg, rosuvastatin 5 or 10 mg, simvastatin 20 or 40 mg, pravastatin 40 or 80 mg, lovastatin 40 mg, fluvastatin XL 80 mg, and pitavastatin 2 or 4 mg daily; or fluvastatin 40 mg twice daily.

Coronary artery disease definitions

Participants were assigned a CAD case or control status using an electronic phenotyping algorithm

that is described in a previous publication (25). In brief, individuals were considered to have CAD if they had a history of coronary revascularization in the EHR, or history of acute coronary syndrome, ischemic heart disease, or exertional angina (ICD-9 codes 410*, 411*, 412*, 413*, 414*) with angiographic evidence of obstructive coronary atherosclerosis (>50% stenosis in at least one major epicardial vessel from catheterization report). CAD controls were defined as individuals without any case criteria or any single encounter or problem list diagnosis code indicating CAD. Two case-control definitions were considered: “general CAD,” in which all individuals, regardless of age, were analyzed, and “premature CAD,” defined as cases and controls younger than 55 (for males) or 65 (for females) years of age.

Statistical analysis

We used a mixed linear model, coding genotypes under an additive model (0 for sequenced non-carriers, 1 for heterozygotes), adjusting for age, age², sex, and the first 5 principal components of ancestry to test for associations between predicted and known pathogenic variants in *LDLR*, *APOB*, and *PCKS9* individually and in aggregate, and maximum EHR-documented LDL-C values. ANOVA with post hoc Tukey honest significant difference tests was used to examine differences in maximum LDL-C values across *LDLR*, *APOB*, and *PCKS9* variant carriers.

We tested for associations between *LDLR*, *APOB*, and *PCKS9* variants in aggregate (coded as above) and “general” and “premature” CAD liability adjusting for age, age², and sex using mixed linear model association analysis. Odds ratios for CAD were estimated using Firth’s penalized likelihood logistic regression (48) adjusting for age, age², sex, and the first five principal components of ancestry. Wald 95% confidence intervals were estimated for odds ratios using standard error estimates back-calculated from p-values from the mixed linear models of association. GCTA v2.1.4 (49) and R version 3.2.1 (R Project for Statistical Computing) were used for all statistical analyses.

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of the board of directors, and stockholder in Regeneron Pharmaceuticals. Pathogenic and predicted pathogenic variants reported in this paper are tabulated in table S2. All variants will be clinically confirmed by the Laboratory of Molecular Medicine (Partners Healthcare) and deposited into ClinVar (www.ncbi.nlm.nih.gov/clinvar/). At the time of publication, 11 of the 35 reported variants had been deposited into ClinVar. Additional information for reproducing and extending the results described in the article is available under a data use agreement with Regeneron Genetics Center LLC and Geisinger Clinic.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6319/aaf7000/suppl/DC1
Fig. S1
Tables S1 to S8

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RESEARCH ARTICLE SUMMARY

CELL FATE DECISIONS

Spatiotemporal antagonism in mesenchymal-epithelial signaling in sweat versus hair fate decision

Catherine P. Lu, Lisa Polak, Brice E. Keyes, Elaine Fuchs*

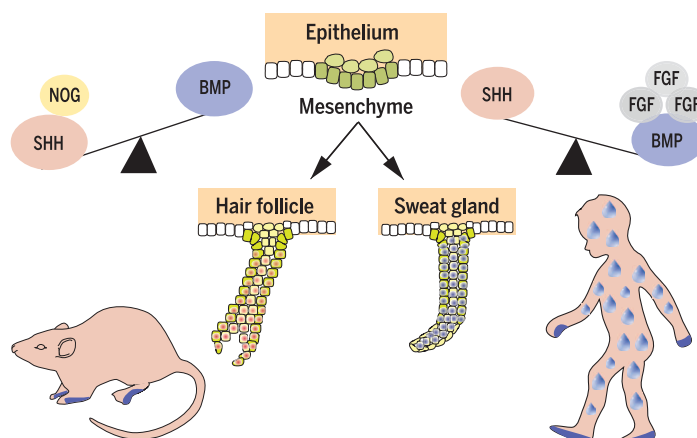
INTRODUCTION: Across the vertebrate kingdom, epithelial appendages—including mammary, sweat, and salivary glands; hair follicles (HFs); teeth; scales; and feathers—begin to form during embryogenesis when WNT signaling instructs progenitors within the epithelial sheet to organize into morphologically similar placodes. Most animals restrict these epidermal appendages to distinct body regions. This paradigm shifted late in mammalian evolution; the dual presence of HFs and eccrine sweat glands (SwGs) in the skin is a recent acquisition of primates.

Classical tissue recombination experiments from the 1960s revealed that mesenchyme directs the divergent downstream events that determine appendage specification and patterning. Relatively little is known about the specific spatiotemporal cross-talk and molecular mechanisms that underlie epithelial fate specification in response to mesenchymal signals. Elucidating the epithelial-mesenchymal cross-talk involved in regional skin appendage specification is integral to understanding how humans have adjusted these mechanisms to endow them with a greater capacity than that of their hairy cousins to live in diverse environments.

RATIONALE: The acquisition of SwGs and their importance in thermoregulation and water balance are underscored by human patients who suffer from the life-threatening condition of lacking SwGs, either from loss in severe burns or from genetic disorders. Conversely, a gain-of-function variant that elevates SwG numbers has been expanding among the Southeast Asian population, where excessive SwGs are desirable. By elucidating the underlying mechanisms that distinguish humans from other mammals in their ability to make both glands and follicles

over their body skin, our findings could pave the way for future therapeutic advances in skin regeneration with dual appendages.

RESULTS: Using mouse as a model, we explored the differences between the back skin mesenchyme, which is only competent to specify HFs, and the foot skin mesenchyme, which can only make SwGs. Using genome-wide analy-



BMP-SHH antagonism specifies SwG versus HF fate. To specify SwGs, mesenchymal-derived BMPs and FGFs signal to epithelial buds and suppress epithelially derived SHH production. Conversely, hair follicles are specified when mesenchymal BMP signaling is inhibited, permitting SHH production. This antagonism is spatially restricted in most mammals but temporally regulated in humans, permitting the presence of HFs (pink), SwGs (purple), or both HFs and SwGs (pink with purple droplet) throughout our body skin.

ses and functional studies, we discovered that just after the formation of morphologically similar epidermal buds, appendage choice is determined through regional skin differences in mesenchymal expression of bone morphogenetic proteins (BMPs). Probing into mechanisms, we showed that when BMPs are elevated in foot skin mesenchyme, BMP signaling is activated in both dermis and epidermis. This triggers a cascade of downstream signaling events. WNT signaling is elevated in the dermis and reduced in the epidermis. Mesenchymal fibroblast growth factors (FGFs)

appear and affect the overlying epithelium. These converging pathways lead to suppression of sonic hedgehog (SHH) in the epithelium.

This BMP:SHH antagonism within the epithelial bud specifies SwG fate and prevents HF fate. Moreover, by manipulating gene expression in vivo at specific developmental stages, we demonstrated that this signaling circuitry acts only within a narrow window of time during mouse embryogenesis. Thus, when SHH is ectopically expressed in foot skin epithelium during the permissive phase, HF-specific gene expression is up-regulated in the epithelial bud, whereas SHH signaling in the mesenchyme stimulates expression of BMP antagonists, further suppressing local BMP signaling and blocking SwG fate. In human skins, this antagonistic interplay of BMP:SHH signaling occurs temporally, in addition to spatially. The first bud waves are specified as HFs, and then a tipping in the balance of BMP:SHH signaling results in the last waves of buds becoming SwGs.

CONCLUSION: Our findings revealed a differential impact of BMP signaling on appendage fate specification that has ancient roots and occurs repeatedly throughout vertebrate evolution. In the evolutionary developmental biology view of integument, chicken scales and mammalian SwGs require BMP signaling to specify their fate, whereas feathers and HFs must suppress it. Our discovery of BMP-SHH antagonism in bud fate choice uncovered additional evolutionary parallels, this time between two even more distantly related epidermal appendages, the mammalian SwG and the fly wing. Our studies provide new insights into how elevated mesenchymal BMP signaling triggers a self-perpetuating molecular cascade that culminates in silencing of SHH signaling to suppress one appendage fate and specify

another. In most mammals, the BMP:SHH antagonism is regulated spatially. Humans, however, have evolved to regulate it temporally, endowing them with greater ability to run marathons and survive in extreme climates. ■

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RESEARCH ARTICLE

CELL FATE DECISIONS

Spatiotemporal antagonism in mesenchymal-epithelial signaling in sweat versus hair fate decision

Catherine P. Lu,¹ Lisa Polak,¹ Brice E. Keyes,^{1*} Elaine Fuchs^{1,2†}

The gain of eccrine sweat glands in hairy body skin has empowered humans to run marathons and tolerate temperature extremes. Epithelial-mesenchymal cross-talk is integral to the diverse patterning of skin appendages, but the molecular events underlying their specification remain largely unknown. Using genome-wide analyses and functional studies, we show that sweat glands are specified by mesenchymal-derived bone morphogenetic proteins (BMPs) and fibroblast growth factors that signal to epithelial buds and suppress epithelial-derived sonic hedgehog (SHH) production. Conversely, hair follicles are specified when mesenchymal BMP signaling is blocked, permitting SHH production. Fate determination is confined to a critical developmental window and is regionally specified in mice. In contrast, a shift from hair to gland fates is achieved in humans when a spike in BMP silences SHH during the final embryonic wave(s) of bud morphogenesis.

Epithelial appendages—including hair follicles (HFs) and teeth as well as mammary, sweat, and salivary glands—begin to form during embryogenesis when Wnt signaling triggers progenitors within the epithelial sheet to organize spatially into morphologically similar placodes. Whereas most mammals restrict the specification of epidermal appendages regionally, HFs and sweat glands (SwGs) coexist throughout much of the skin of primates. The acquisition of SwGs and their importance in thermoregulation are underscored by humans who suffer from a life-threatening condition when SwGs are eliminated, either from loss in severe burns or from the genetic disorder hypohidrotic ectodermal dysplasia (HED).

Patients with HED display mutations in genes encoding proteins such as ectodermal dysplasia antigen (EDA), the EDA receptor (EDAR), and WNT10a, a ligand for Wnt signaling. These findings have illuminated a critical role for these pathways in controlling the development of a number of epidermal appendages, including SwGs and coarse hairs (1, 2). Conversely, an EDAR gain-of-function variant has been expanding among the Southeast Asian population, where excessive SwGs are desirable because of the hot and humid climate of that region (3). In this regard, both Wnt and EDA/EDAR pathways appear to function in promoting placode formation, increasing the density of SwGs and several other appendage types (4).

Classical tissue recombination experiments have revealed that mesenchyme plays a critical

role in dictating the divergent downstream events that determine appendage selection (5, 6). For example, salivary mesenchyme combined with mammary epithelium generates an epithelial morphology resembling salivary glands (7), and regardless of its regional origin, embryonic chick epidermis develops feathers if combined with feather-forming dermis but develops scales if the epidermis is exposed to scale-forming dermis (8). Similarly, rabbit corneal epithelium generates either HFs or SwGs depending on whether it is exposed to the mesenchyme of dorsal back skin or ventral foot skin, respectively (9). Despite the existence of many such examples, relatively little is known about the specific spatiotemporal cross-talk and molecular mechanisms that underlie epithelial fate specification in response to mesenchymal exposure.

Regional differences in BMP pathway genes in skin mesenchymes

To understand how epithelial fate is specified by mesenchymal signals, we began by exploiting the fact that in mice, dorsal back skin supports only HF morphogenesis, whereas eccrine SwG morphogenesis is restricted to ventral foot skin. We first pinpointed the appearance of WNT-induced epidermal placodes at these body sites, reasoning that the underlying mesenchymes become competent to specify the differential fates of these placodes. We then carried out genome-wide, high-throughput RNA sequencing (RNA-seq) on body site-specific dermal mesenchymes at the embryonic ages when their respective placodes emerge [embryonic day 14.5 (E14.5), dorsal back skin; E17.5, ventral foot skin] (Fig. 1A).

Comparative bioinformatics revealed that bone morphogenetic protein (BMP) signaling pathway genes were significantly enriched [$\geq 2\times$, $P <$

0.05, false-discovery rate (q value) < 0.05] among the transcripts [with fragments per kilobase per million mapped reads (FPKM) > 1] in ventral foot skin versus dorsal back skin dermis (Fig. 1B). Of these, the BMP5 ligand stood out for its regional-specific expression, confirmed with quantitative polymerase chain reaction (PCR) (Fig. 1C). Also surfacing in our differential transcriptome analysis were *Bmpr1a*, encoding a key component of the BMP receptor kinase, and *Id1* and *Msx1*, two downstream targets of its activated transcriptional effectors, phospho-SMAD1/5/8 (Fig. 1B).

Anti-phospho-SMAD1/5/8 immunofluorescence confirmed the marked differential elevation in BMP signaling within ventral foot skin versus dorsal back skin during the fate-permissive state of their epithelial buds (Fig. 1D). However, there was also strong nuclear immunolabeling in the overlying SwG buds, not seen in HF buds. *Bmpr1a* transcripts were also markedly elevated in SwG-permissive epidermis, relative to HF-permissive epidermis (Fig. 1E). We therefore examined BMPs and BMP inhibitors in both mesenchyme and epidermis. Despite expression of other BMPs and BMP inhibitors in both mesenchyme and epidermis, only mesenchymal BMP5 exhibited a regional-specific expression that might account for the striking difference in BMP signaling that we observed between SwG and HF buds (Fig. 1C).

Mesenchymal-epithelial BMP signaling in specifying SwGs

If paracrine mesenchymal-epithelial BMP signaling in foot skin occurs, as our data suggest, and if this cross-talk is physiologically important, then loss of mesenchymal *Bmp5* should affect overlying SwG development. Ablating *Bmp5* significantly reduced SwG numbers in ventral foot skin and resulted in decreased epithelial BMP signaling (pSMAD1) (Fig. 1F and fig. S1A). Although the effect was robust, SwGs still developed and BMP signaling was still seen in both mesenchymal and epithelial compartments of ventral foot skin (fig. S1A), suggesting that other mesenchymal BMPs may partially compensate for the loss of BMP5. To test this possibility and assess the full functional relevance of the elevated BMP signaling in the footpads, we used K14-Cre mice to conditionally ablate *Bmpr1a* in embryonic skin epidermis. ImmunopSMAD1/5/8 labeling confirmed that BMP signaling was completely abrogated in the epithelium, whereas it was still sustained as expected in the underlying mesenchyme (fig. S1B). Under these conditions, no SwGs were generated (fig. S1C).

Instead of forming a coiled gland, the *Bmpr1a*-null epithelial down-growths in the ventral foot pads enveloped the associated dermal mesenchyme and assumed a morphology, biochemistry, and proliferative status more typical of HF bulbs (Fig. 1G and fig. S1D). By postnatal day 5 (P5), K17, LHX2, and P-cadherin—hallmarks of HFs (10) and not expressed by SwGs—were all ectopically expressed in *Bmpr1a*-null foot skin epithelial down-growths. In contrast, smooth muscle

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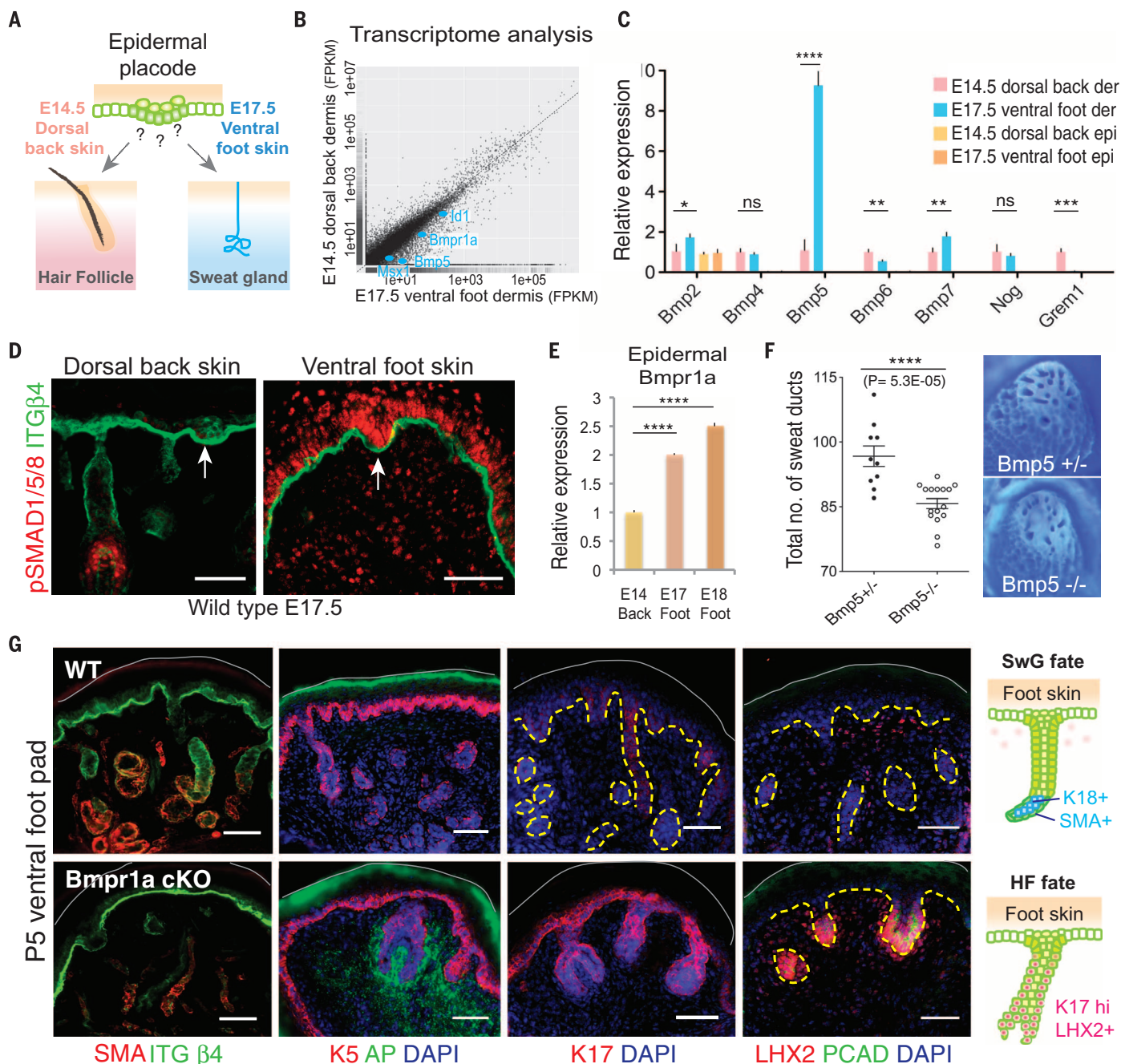


Fig. 1. Mesenchymal-epithelial BMP signaling is required for sweat gland fate. (A) Schematics of spatiotemporally distinct development of mouse sweat glands and hair follicles. (B) Scatter plot of RNA-seq analysis of E14.5 dorsal back skin and E17.5 ventral foot skin dermis. *Bmpr1a*, *Bmp5*, *Id1*, and *Msx1* (targets of BMP signaling) are enriched in ventral foot skin dermis (blue dots). (C) Quantitative PCR for various BMP ligands and inhibitors in E14.5 dorsal back skin (HF-permissive) and E17.5 ventral foot skin (SwG-permissive) epidermis and dermis. Shown is a strong up-regulation of *Bmp5* in mouse foot skin dermis. (D) Immunofluorescence of phospho-SMAD1/5/8 in dorsal back and ventral foot skins. Arrows denote hair and sweat placodes, respectively. Scale bars, 50 μ m. (E) Quantitative PCR for epidermal *Bmpr1a* mRNAs at indicated embryonic

stages and regional skins. (F) (Left) Quantification of sweat ducts in adult *Bmp5* heterozygous and null mice. $n = 10$ and 16 foot skin samples, respectively. (Right) Representative images of adult foot pad epidermis stained with Nile blue A in order to visualize sweat ducts. (G) Immunofluorescence images of foot pads of wild type (WT) and *Krt14-Cre; Bmpr1a* conditional knockout (cKO) at P5. SMA, smooth muscle actin, a myoepithelial glandular marker; K5, keratin 5, which marks the basal layer of epidermal progenitors; ITGβ4, integrinβ4, which marks the basal surface of the epidermis; AP, alkaline phosphatase, a marker for HF dermal papillae; K17, LHX2 and PCAD, markers of the HF. Gray solid lines denote skin surface. Dashed lines indicate dermo-epidermal border. Scale bars, 50 μ m. (Right) Schematics of SwG fate and HF fate in the foot skin.

actin, featured by the myoepithelial cells of the gland, was absent (Fig. 1G). Further reflective that an epithelial fate switch had occurred, we observed condensation of alkaline phosphatase-

positive mesenchymal cells into a structure resembling a dermal papilla (DP), the cluster of mesenchymal cells associated with the HF that are essential for its growth and maintenance

(17). These data not only document the physiological relevance of elevating epithelial BMP signaling in developing SwGs but also suggest that paracrine cross-talk between mesenchymal

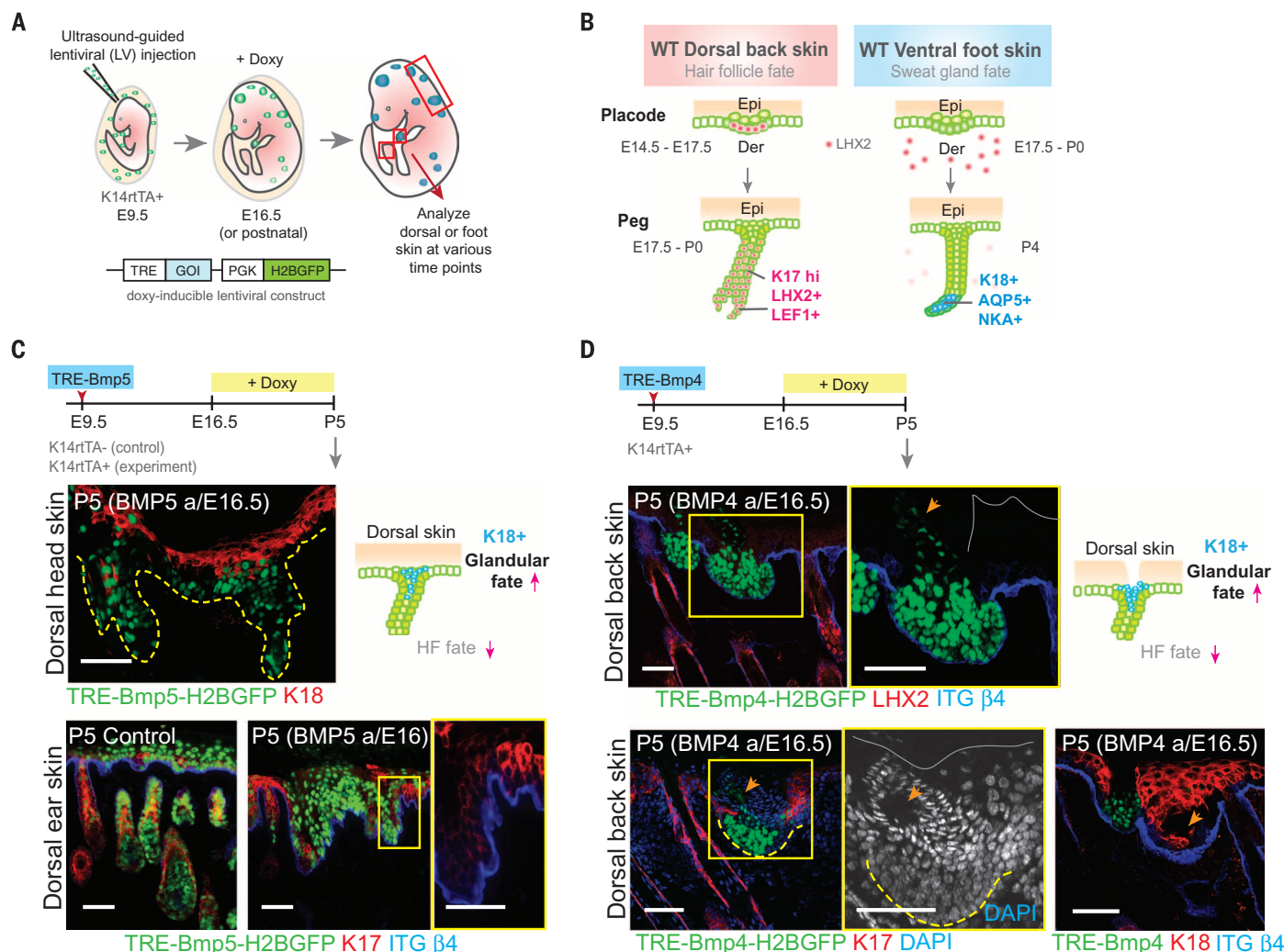


Fig. 2. Ectopic elevation of BMP signaling in dorsal skin elicits a HF: glandular fate shift. (A) Schematic of ultrasound-guided in utero lentiviral injections and doxycycline (Doxy)-inducible ectopic expression system. *K14rtTA* embryos express the Doxy-inducible transactivator beginning at ~E13.5. (B) Schematic of wild-type hair and sweat placode and peg stages. HF and SwG markers are shown in pink and blue, respectively. (C and D) Timeline of lentiviral injection, BMP5 (C) and BMP4 (D) induction and analyses. Im-

muno fluorescence images of dorsal skin show loss of HF markers and ectopic expression of glandular luminal cell marker *K18*. Lentiviral injections were performed at least two times, and two to four animals were collected and analyzed at each time point. Boxed areas are shown in higher magnification at right. Gray solid lines mark the skin surface. Dashed lines indicate dermo-epidermal border and arrows indicate ductal structures associated with BMP4-expressing epidermal cells. Scale bars, 50 μ m; except (C) lower right, 25 μ m.

BMPs and BMP receptor^{high} epithelial buds is critical for SwG development.

BMP signaling can cause a hair follicle to glandular fate switch

To further test whether these regional differences in BMP signaling are key to SwG versus HF appendage fate, we generated lentiviruses harboring a *PGK-H2BGFP* marker and doxycycline-inducible transgenes encoding BMPs and BMP inhibitor. We then used an ultrasound-guided in utero injection system (12) to introduce lentivirus into the amniotic sacs of living E9.5 *K14rtTA* mouse embryos (Fig. 2A). Within 24 hours, the transgene and green fluorescent protein (GFP) marker selectively and stably integrate into the host genome of epidermal progenitor cells and their progeny (12).

Transgene expression can then be controlled by the timing of doxycycline administration.

We first tested the consequences of elevating BMPs in dorsal hairy skin. After transducing with *TRE-Bmp5*, we administered doxycycline at E16.5 of gestation. Transgene expression was robust by E17.5 during permissive phases of sweat buds and final waves of HF buds (Fig. 2, B and C). By analyzing at P5, we learned that BMP5 dramatically perturbed back skin HF morphogenesis, and HF markers were lost (Fig. 2C). A similar block in HF morphogenesis was seen upon activation of *TRE-Bmp4* in embryonic back skin epidermis (Fig. 2D). Elevating BMP levels not only blocked HF morphogenesis in dorsal back skin but also ectopically induced glandular and ductal-like structures, accompanied by expression of glan-

dular keratin, *K18* (Fig. 2D). Together with our loss-of-function data, these results suggest that collective BMP levels, rather than specific ligand, affect fate choice.

BMP inhibition acts within a critical window to cause SwG to HF fate switch

We next examined the consequences of inhibiting BMP signaling throughout the ventral foot skin by ectopically expressing the secreted BMP inhibitor NOGGIN. Prior studies on back skin revealed a role for NOGGIN in promoting HF fate (13), and it was previously postulated that ectopic NOGGIN might trigger “transdifferentiation” of SwGs into hairs (14). Our findings raised the additional possibility that by intercepting mesenchymal BMPs, NOGGIN might act by blocking

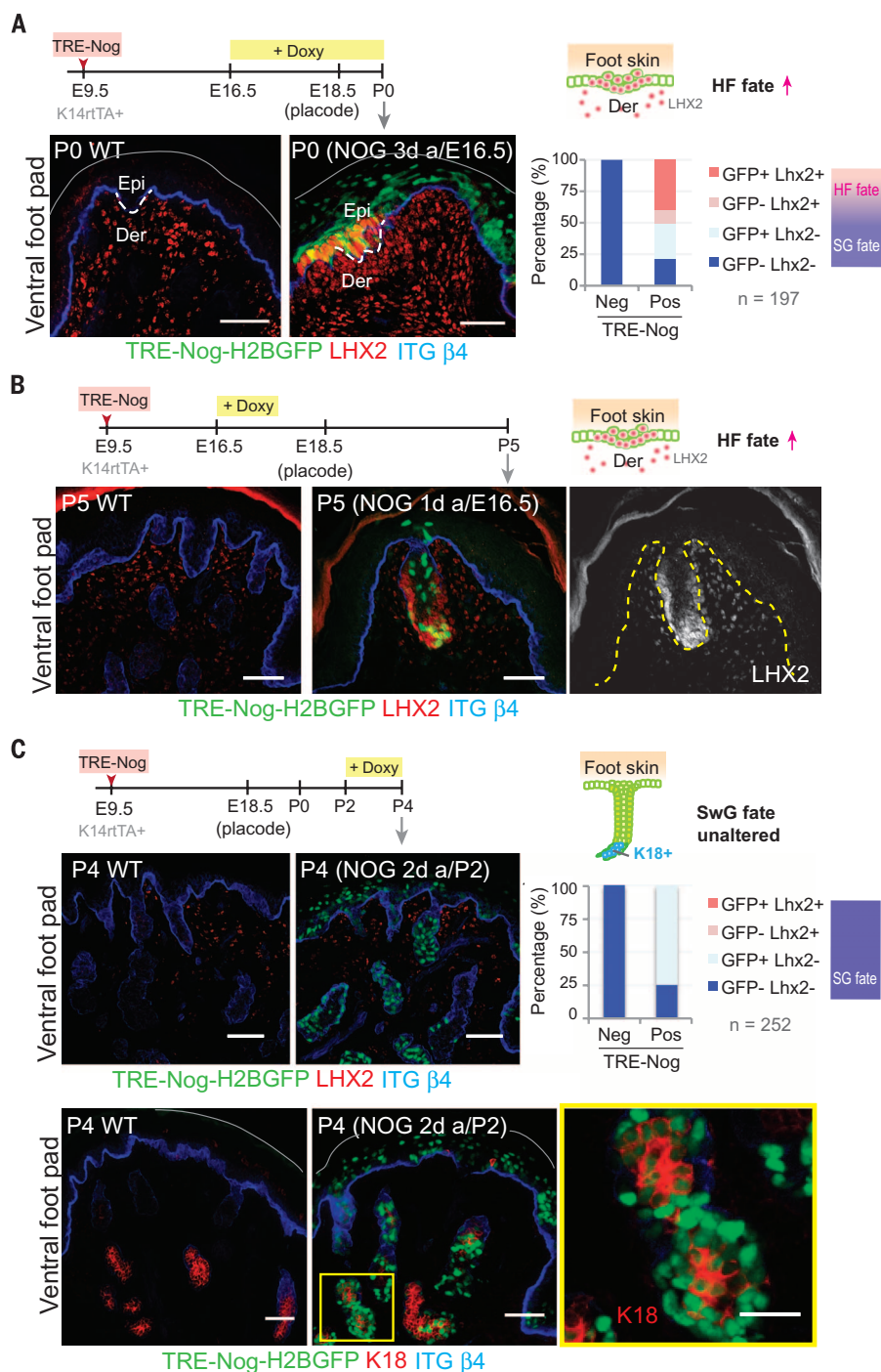


Fig. 3. Ectopic inhibition of BMP acts during a critical window to suppress SwG fate in the foot skin. (A to C) Timeline of LV injection, NOGGIN induction, and analyses. (A) Immunofluorescence images and quantification of LHX2⁺ and LHX2⁻ cells in the LV-infected and uninfected epidermis from three different foot pads (cell number as indicated). When BMP is inhibited during early stages of epithelial bud formation, a switch from SwG to HF fate occurs, reflected by the appearance of LHX2 in the epidermis. (B) Immunofluorescence images of LHX2. A brief pulse of NOGGIN expression at the placode stage is sufficient to cause a fate change that lasts postnatally. (C) Immunofluorescence images and quantification of LHX2⁺ and LHX2⁻ cells in the LV-infected and uninfected epidermis from three different foot pads (cell number as indicated). Inhibiting BMP after the placode stage fails to elicit a fate change, as reflected by the sustained expression of SwG luminal cell marker K18 in the NOGGIN⁺ ventral foot skin. Lentiviral injections were performed at least two times, and two to four animals were collected and analyzed at each time point. Gray solid lines mark the skin surface. Dashed lines indicate dermo-epidermal border. Boxed area is shown in higher magnification at right. Scale bars, 50 μ m and (bottom far right) 25 μ m.

signaling directly to the epithelium, redirecting foot skin buds to a HF fate. To test this possibility, it was necessary to control NOGGIN expression.

We first induced a TRE-*Noggin* transgene at E16.5 of embryogenesis just before sweat bud formation. Soon thereafter, the epithelial foot skin buds showed signs of HF morphogenesis, including LIM/homeobox protein (LHX2) expression (Fig. 3A). Quantification revealed that even at these early times after *Noggin* activation, ~50% of transduced (GFP⁺) cells were already LHX2⁺, an early HF fate indicator. These changes occurred before signs of dermal condensation.

A short pulse of NOGGIN expression at this critical placode stage was sufficient to trigger these events, which then lasted postnatally, as evidenced by the LHX2-positive hair pegs within P5 foot skin (Fig. 3B). In contrast, when we waited until P2 to initiate NOGGIN expression, no adverse effects were seen in the foot skin (Fig. 3C). Glands were still formed and expressed only SwG and not HF markers, without any trace of early HF marker LHX2 in NOGGIN-expressing cells. Together, these results extend earlier findings of Plikus *et al.* (14) but add three key new points: First, BMP activation in back skin or BMP inhibition in foot skin potentially affects SwG versus HF fate determination. Second, the impact of BMP signaling on SwG fate choice occurs exclusively within a narrow temporal window when ventral foot skin buds appear. Third, the BMP-mediated changes in the epithelium precede those in mesenchyme, underscoring the importance of mesenchymal-epithelial cross-talk and offering molecular insights into the classical tissue recombination experiments.

BMP signaling enhances mesenchymal but dampens epithelial Wnt signaling

Canonical Wnt signaling, mediated through its effector β -catenin and its transcriptional coactivator lymphoid enhancer-binding factor 1 (LEF1), is essential for placode formation irrespective of appendage type (15–18). Indeed, epidermal-specific loss of β -catenin abrogated sweat bud formation (Fig. 2A) (18). Cross-talk at the epidermal-dermal border involving Wnt signaling is also well established (19). However, we were curious to know how the subsequent elevation in BMP signaling throughout the ventral foot skin would affect this cross-talk; although BMP inhibition is known to elevate Wnt signaling and stabilize LEF1 in hair buds (20, 21), back skin explant studies have suggested a positive role for BMP signaling in mesenchymal *Left* expression (22). If these prior studies are functionally relevant, the heightened BMP signaling throughout the ventral foot skin should skew the Wnt cross-talk at the epidermal-dermal border, elevating mesenchymal Wnt signaling and dampening epithelial Wnt signaling downstream from placode formation.

Indeed, during the bud-permissive phase of ventral foot skin, WNT reporter (*Axin2-LacZ*) activity was markedly stronger in the mesenchyme than in epithelium (Fig. 4A). Closer inspection revealed its presence in the epithelial placodes, but as buds developed, it became even weaker

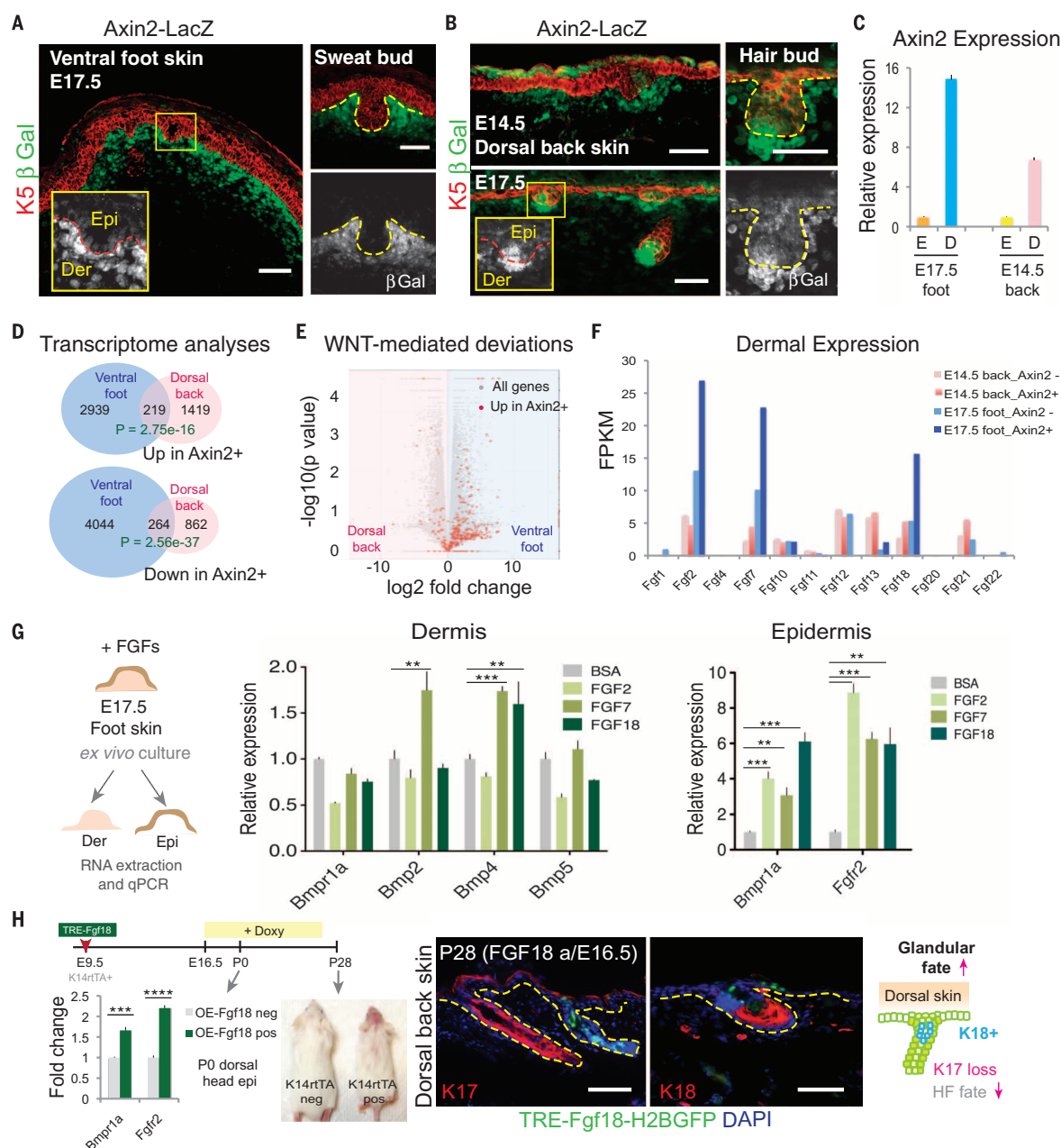


Fig. 4. WNT responsiveness is elevated in mesenchyme and dampened in epithelium of BMP-high ventral foot skin, leading to dermal and epidermal changes in fate determinants of SwG-permissive skin.

(A and B) Axin2-LacZ (WNT) reporter expression reveals a sharpened epithelial-mesenchymal WNT differential in ventral foot skin (E17.5) (A), compared with dorsal back skin (E14.5 and E17.5) (B). Boxed areas highlight marked differences in Wnt signaling between sweat and hair buds (from E17.5 foot skin and back skin, respectively). Epi, epidermis; Der, dermis. Dashed lines indicate dermo-epidermal border. Scale bars, 50 μ m. (C) Quantitative PCR for Axin2 expression in the epidermis (E) and dermis (D) of E17.5 foot skin and E14.5 back skin, confirming the sharpened epithelial-mesenchymal WNT differential in ventral foot skin (E17.5), compared with dorsal back skin (E14.5). Dermal values are normalized to the corresponding epidermis. (D) Venn diagrams summarizing transcriptome analyses of genes up-regulated or down-regulated in Axin2-LacZ-positive ventral compared with dorsal dermis. (E) Volcano plot showing all transcripts that are log2-fold changed in dorsal versus ventral dermis. Transcripts greater than fivefold changed between Axin2-positive and Axin2-negative cells of each dermal

population are labeled in red. WNT-responsive genes are generally more highly expressed in ventral foot skin relative to dorsal back skin dermis. (F) Expression of FGF ligand family member transcripts in WNT-responding and -nonresponding fibroblasts from dorsal and ventral dermis at their respective embryonic stages when epidermal placodes emerge. Shown are strong up-regulation of *Fgf2*, *Fgf7*, and *Fgf18* in Axin2-LacZ-positive versus -negative foot dermis. (G) Ex vivo effects of FGFs on E17.5 foot skin. (Left) After 16 to 20 hours of treatment, dermis and epidermis were manually separated and analyzed by means of quantitative PCR (middle and right, respectively) for transcripts indicated. Shown is a FGF-mediated *Bmp* elevation in dermis; *Bmp1a* and *Fgf2* in epidermis. *n* = 6 to 8 ventral foot skin explants from three embryos. (H) Timeline of lentiviral injection, TRE-FGF18 induction, and analyses of dorsal skin. Quantitative PCR shows FGF18-induced up-regulation of *Bmp1a* and *Fgf2* in vivo. Hair loss is visible in the highly transduced head skin as a result of ectopic FGF18 expression. (Right) Immunofluorescence images and diagram showing loss of HF marker K17 and gain of glandular marker K18 upon FGF18 induction. Dashed lines indicate dermo-epidermal border. Scale bars, 50 μ m.

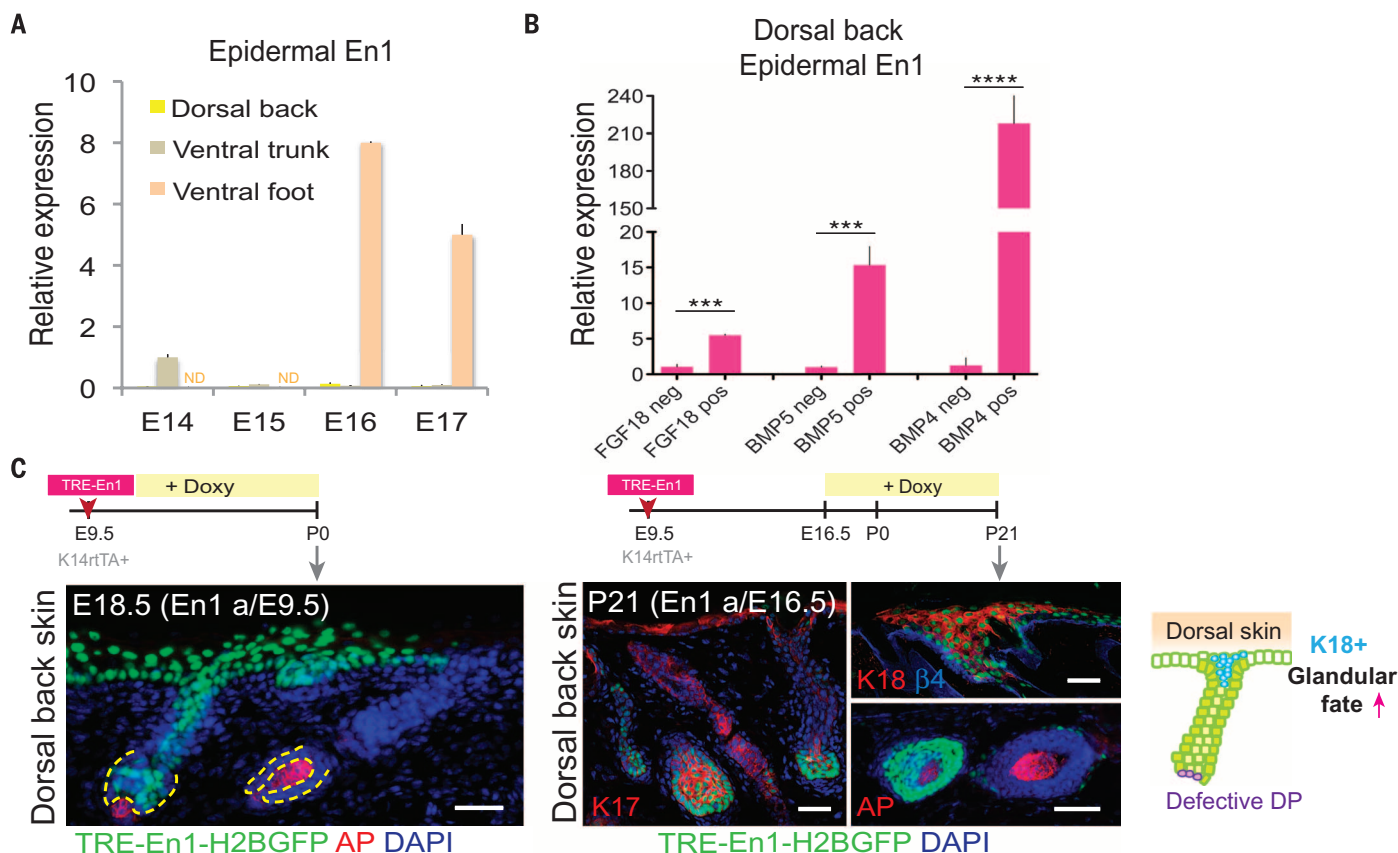


Fig. 5. *Engrailed-1* is expressed during SwG fate specification in ventral foot skin and interferes with DP formation when ectopically expressed in dorsal back skin. (A) *En1* quantitative PCR of dorsal back, ventral trunk, and ventral foot epidermal mRNAs during placode-permissive stages. (B) *En1* is elevated in dorsal back skin epidermis of ectopic *Fgf18*-, *Bmp5*-, and *Bmp4*-expressing embryos after 3 days of doxy-induction. (C) Timeline of lentiviral

injection, *TRE-En1* induction, and analyses of dorsal back skin after ectopic expression. Immunofluorescence images for AP (alkaline phosphatase, a marker for DP) and K18 (a marker for glandular fate). There was diminished DP and ectopic induction of K18 in *En1*-expressing epidermal down-growths of dorsal back skin. Dashed lines indicate dermo-epidermal border. Scale bars, 50 μ m.

(Fig. 4A) and only reappeared in sweat epithelium when sweat ducts were formed (fig. S2B). In contrast, during the bud-permissive phase of dorsal back skin, WNT reporter activity was present in both mesenchyme and epithelium (Fig. 4B) (23). Similarly, nuclear LEF1, a key canonical Wnt effector, showed nuclear LEF1 transiently in SwG buds and diminished LEF1 in down-growths of ventral foot skin, whereas in dorsal back skin, LEF1 was high in the epithelial buds and sustained at the leading front of the down-growths, where it was seen in both the epithelium and the DP (fig. S3A). Quantification by means of quantitative PCR confirmed that the Wnt gradient was sharper in the ventral foot skin than in the dorsal back skin during the bud-permissive phase (Fig. 4C).

During the time when ventral foot skin was permissive for sweat bud development, the elevation in BMP and Wnt signaling was accompanied by other features that distinguished this mesenchyme from its dorsal counterpart. Reflecting the elevation in BMP signaling at this time, alkaline phosphatase exhibited a broad mesenchymal pattern similar to LEF1 (fig. S3B). LHX2, which in back skin is restricted to the WNT-high

cells of the hair bud (10), was also mesenchymally expressed in permissive ventral foot skin dermis (fig. S3C). Postnatally, as the window of sweat bud fate specification waned, so too did all of these features (Fig. 1G and fig. S3).

Elevated Wnt signaling through BMP signaling contributes to divergent mesenchymes

Probing further into how differences in mesenchymal Wnt signaling at the epidermal-dermal border regionally influence the transcriptional landscape of body-site mesenchymes that support distinct bud fates, we transcriptionally profiled the *Axin2*-positive and *Axin2*-negative dermal cells after their fluorescence-activated cell sorting (FACS) purification from E17.5 ventral foot skin and E14.5 dorsal back skin (fig. S4). RNA-seq analyses revealed a small percentage ($\leq 10\%$) of WNT-responsive genes that are shared between glandular and follicular-competent mesenchyme (Fig. 4D). Volcano plot analysis further revealed that transcripts greater than fivefold up-regulated in the *Axin2*-positive versus *Axin2*-negative dermal subfractions were distributed away from the midline (Fig. 4E), suggesting that

elevated WNT activation through BMP signaling contributes to divergence between glandular and follicular-competent mesenchymes.

Axin2-reporter and Gene Ontology (GO)-term analyses revealed that although Wnt pathway genes were enriched in WNT-receiving cells of both foot and back skin dermis, a number of differences emerged (fig. S5A). *Wnt5a* and *Wnt10a* transcripts more highly expressed in foot skin than back skin dermal cells (fig. S5B): WNT5A is known to induce *Krt9* (24), encoding a signature keratin of palmoplantar epidermis, and WNT10A is the only known Wnt ligand whose mutations have been implicated in human HED (2, 25). However, among the family of BMP ligands, none showed changes between the *Axin2*⁺ and *Axin2*⁻ population (fig. S5C), which is consistent with the notion that BMP signaling is upstream of Wnt signaling in skin mesenchyme (22).

Wnt signaling elicits elevated FGF signaling in foot skin mesenchyme

There was a difference in mRNAs encoding fibroblast growth factor (FGF) ligands and other Ras-mitogen-activated protein kinase (MAPK) pathway members. In particular, *Fgf2*, *Fgf7*, and *Fgf18* were

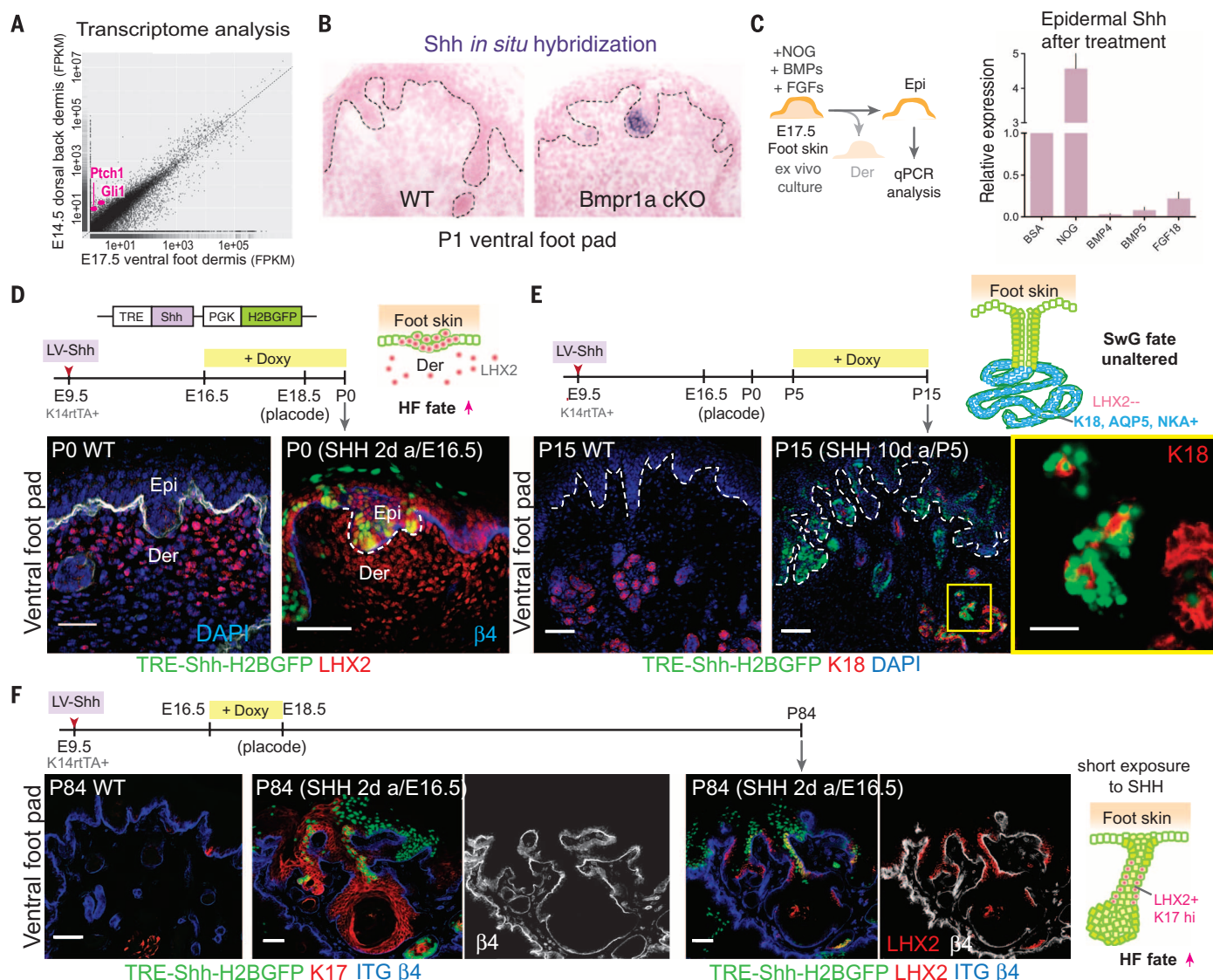


Fig. 6. SHH signaling is suppressed in SwG buds and upon ectopic induction and is only effective within a critical window in switching sweat to hair fate. (A) Scatter plot of RNA-seq transcriptome analyses from E14.5 dorsal back skin and E17.5 ventral foot skin dermis. Targets of SHH signaling, *Gli1* and *Ptch1*, are enriched in dorsal versus ventral dermis (pink dots). (B) *Shh* in situ hybridizations of ventral foot pads of WT and *Bmpr1a* cKO P1 pups, revealing the role of BMP in suppressing *Shh* in SwG-buds. (C) Ex vivo culture of E17.5 foot skin with addition of BMP-inhibitor, NOGGIN, BMPs, or FGF18. Epidermal *Shh* expression was analyzed by means of quantitative PCR. $n = 6$ to 8 foot skin samples analyzed from three embryos per experiment. (D) Timeline of lentiviral injection, *Shh* induction, and analyses. Immunofluorescence images showing that when induction occurs at the epithelial bud stage, hair bud marker

LHX2 is ectopically induced in the foot pad epidermis, which is reflective of a switch from SwG to HF fate. Dashed line indicates dermo-epidermal border. Scale bar, 50 μ m. (E) Timeline of lentiviral injection, *Shh* induction, and analyses. Immunofluorescence images showing that when induction occurs after the epithelial bud stage, appendage fate is unaltered in the ventral foot skin. Boxed area is shown in higher magnification at right. Scale bar, 50 μ m and (far right) 25 μ m. (F) Timeline of lentiviral injection, *Shh* induction, and analyses. Immunofluorescence images showing that short exposure to SHH during foot skin epithelial bud formation is sufficient to cause long-lasting effects on fate change and expression of HF markers K17 and LHX2. Dashed lines indicate dermo-epidermal border. Scale bars, 50 μ m.

more highly transcribed in Wnt-responsive ventral foot skin dermis than in any other dermal fraction examined (Fig. 4F and fig. S5A). We therefore turned to addressing whether FGFs might contribute to sweat fate specification.

To assess FGF functional relevance, we exposed E17.5 foot skin explants for 16 to 20 hours with each of these FGFs and then separated dermis and epithelium and analyzed the transcriptional consequences. FGFs showed little or

minor effect on the dermal expression of BMP ligands or BMP receptor (Fig. 4G, middle). In contrast, their effects on epidermal expression of *Bmpr1a* and *Fgf2* were appreciable (Fig. 4G, right). Although these FGFs are known to activate different receptors and elicit distinct molecular effects in different contexts, their action on these epidermal receptors was similar. Together, these findings suggest that FGFs are selectively up-regulated in BMP-hi and Wnt-

receiving foot skin mesenchyme at the dermo-epidermal border and act directly on the overlying epithelium to sensitize it to paracrine-generated BMPs and FGFs. Indeed, when we transduced embryos with TRE-*Fgf18* and activated FGF18 expression during the HF-permissive phase of dorsal skin, *Bmpr1a* and *Fgf2* were up-regulated (Fig. 4H). In addition, like BMP5 and BMP4, ectopic FGF18 caused hair loss in dorsal head skin, suppressed expression of HF marker K17,

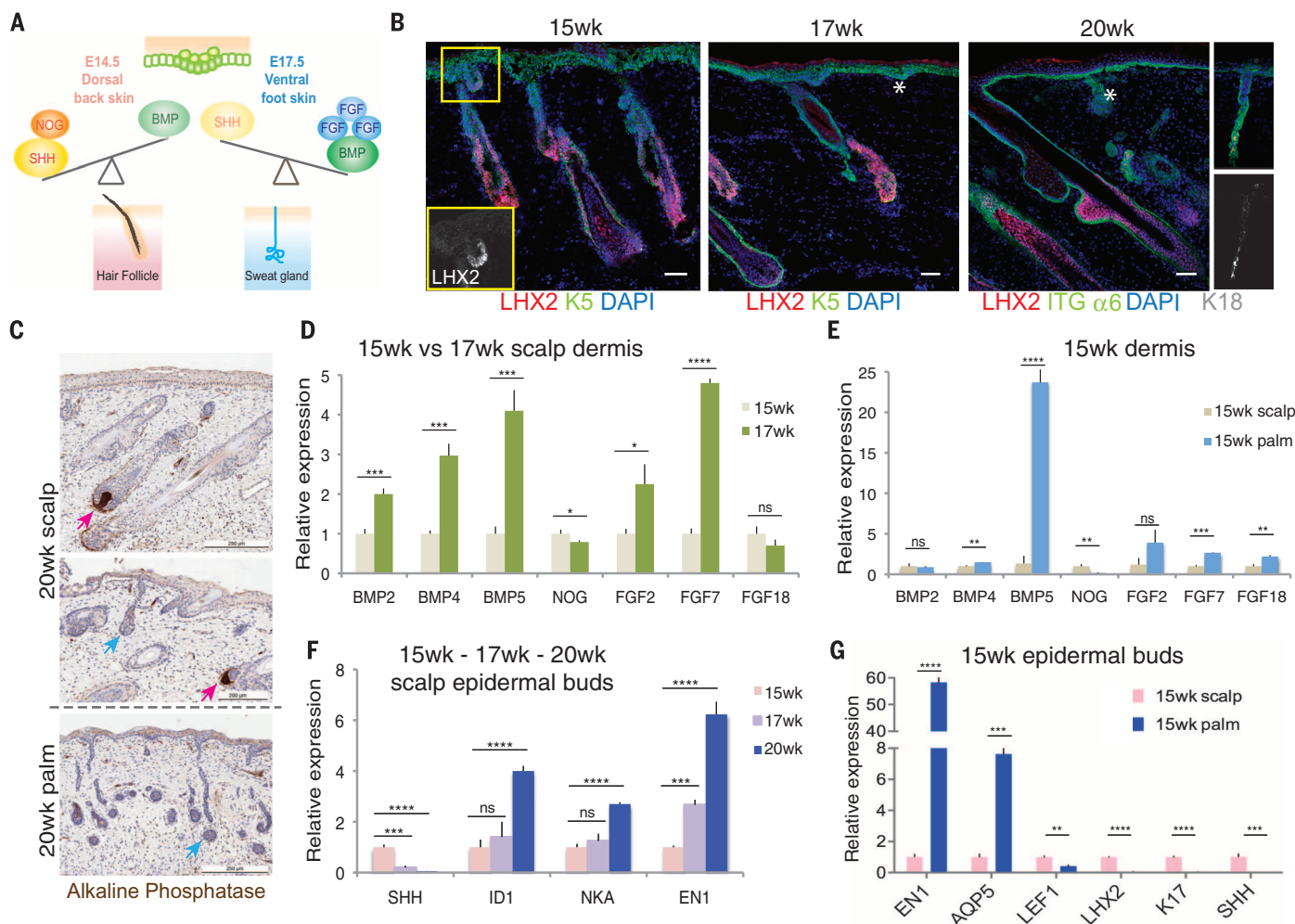


Fig. 7. Human dorsal skin temporally shifts from BMP^{lo}FGF^{lo}SHH^{hi} to BMP^{hi}FGF^{hi}SHH^{lo} during embryogenesis, yielding HF and SwG coexistence. (A) Model showing differences in the balance between BMP/FGF and SHH signaling specify the fate of HFs or SwGs. (B) Immunofluorescence images of human fetal dorsal scalp skin at 15, 17, and 20 weeks of gestation. Boxed area shows emerging LHX2⁺ HF buds. Stars indicate LHX2^{neg} placode and bud. HFs and SwG buds are distinguished by hair bud-specific marker LHX2. (Far right) Appearance of K18-positive maturing sweat gland by 20 weeks. Scale bars, 100 μ m. (C) Histology of dorsal scalp skin and ventral palm skin, costained for alkaline phosphatase activity to identify dermal papillae (pink arrows), absent in sweat buds and glands (blue arrows). Visible is coexistence

of glands and HFs in dorsal scalp skin but only SwGs in palm skin. Scale bars, 200 μ m. (D to G) Quantitative PCR of transcripts isolated from dermis and epithelial buds of scalp skin and palm skin, at ages indicated. (D) Increase in BMP and FGF transcripts in scalp dermis between 15 and 17 weeks. (E) High level of BMP5 expression in 15-week ventral palm (SwG only) versus 15-week dorsal scalp (HF only) dermis. (F) Early decline of SHH production and later increase in BMP signaling (ID1) and SwG markers (NKA) in emerging epithelial buds between 15 and 20 weeks. Also shown is the emergence of EN1 in dorsal scalp skin, which contrasts with mice, where EN1 is confined to ventral foot skin. (G) At 15 weeks, SwG markers are already high in palm but not in scalp epidermis.

and induced expression of glandular marker K18, altogether acting toward glandular fate.

Expression of *Engrailed* during SwG fate specification: Parallels to fly wing development

The BMP-induced sharpened differential that we observed in Wnt signaling at the dermo-epidermal border of permissive ventral foot skin was reminiscent of *Drosophila* wing development, in which strong Wnt signaling cells are juxtapositioned with low-Wnt signaling cells, a condition that in turn was necessary to sustain expression of the transcription repressor *Engrailed* specifically in the low-Wnt-signaling cells (26). We found that *En1* was highly expressed in mouse

ventral epithelium during the SwG fate-permissive stage of foot skin but not in the dorsal epithelium during the HF fate-permissive stage of back skin (Fig. 5A). *En1* was also expressed in early ventral trunk (belly) skin, and its expression waned after E14.5 (Fig. 5A). This timing coincided with the completion of mammary gland fate specification (E13.5) and was just before hair follicle fate specification (E15.5) in the belly skin.

These data point to a complex circuitry triggered by BMP elevation in ventral foot skin mesenchyme that enhances differential Wnt signaling to reach a permissive threshold to result in *En1* expression. In agreement with this hypothesis, TRE-*Bmp5* and TRE-*Bmp4* activation resulted in a dramatic up-regulation of *En1* in dorsal skin

epidermis, as did TRE-*Fgf18* (Fig. 5B). Moreover, when *En1* was ectopically induced, it resulted in transformations consistent with a more glandular fate, including diminished DP and K18 induction (Fig. 5C). Together, these data suggest a role for epidermal ENGRAILED-1 in promoting the SwG fate; we found this noteworthy because mice lacking *Engrailed-1* dorsalize the ventral skin (27), and *Engrailed-1* heterozygote mutant mice display fewer SwGs in their ventral foot skin (28).

All roads lead to suppression of SHH signaling in SwG-permissive skin

Studies on the HF have emphasized the role of epithelial sonic hedgehog (SHH), downstream

of Wnt signaling, in stimulating mesenchymal clustering to form the *Noggin*-expressing DP (29, 30). Because sweat buds display reduced epithelial Wnt signaling and also lack this mesenchymal cluster, we posited that in sweat buds, *Shh* is silenced, which in turn should be reflected in the failure of foot skin dermis to activate downstream SHH-signaling target genes. Our RNA-seq analyses showed that indeed, *Gli1* and *Ptch1*, the classic downstream target genes of SHH signaling, are highly enriched in dorsal back skin (HF-competent) versus ventral foot skin (SwG-competent) dermis (Fig. 6A). Quantitative PCR confirmed the strong expression of *Shh* in E14.5 dorsal epidermis relative to ventral foot skin epidermis (fig. S6A).

Because *Bmpr1a*-null foot skin buds displayed signs of mesenchymal cell clustering (Fig. 1G), we hypothesized that *Shh* expression must rely not only on epithelial Wnt signaling (21, 30) but also on inhibition of mesenchymal BMP signaling to the epithelial buds. In other words, when mesenchymal BMPs actively signal to the epithelial buds, as in the case of ventral foot skin, *Shh* expression must be suppressed. In situ hybridizations and quantitative PCR showed that *Shh*, although not detected in wild-type ventral foot skin, was indeed activated in *Bmpr1a*-null ventral foot skin buds in vivo, as well as in E17.5 foot skin explants exposed to NOGGIN (Fig. 6, B and C). In contrast, exposure of foot skin explants to additional BMPs further suppressed already low expression of *Shh* (Fig. 6C). Furthermore, elevating NOGGIN in dorsal back skin resulted in higher activation of *Shh*, as well as HF markers *Lhx2* and *K17* (fig. S6B).

Shh repression in ventral foot skin buds seemed also to be influenced by FGF-MAPK signaling, perhaps through its ability to sensitize buds to BMP signaling (Figs. 4G and 6C). In particular, FGF18 seemed to have the strongest effect on *Shh* repression as shown in explants and in vivo when activated in dorsal back skin (Fig. 6C and fig. S6, C and D). The combinatorial effect of FGFs in *Shh* suppression could be partially rescued by MAPK inhibitor SB203580 (fig. S6D). These results correlated with GO-term analyses of WNT-responsive changes, revealing that the MAPK pathway is positively enriched in ventral foot skin but negatively enriched in dorsal back skin (fig. S5A).

Our data on FGFs further bolstered the importance of mesenchymal-to-epithelial signaling circuits for *Shh* suppression in foot skin. That said, Wnt signaling is required and upstream of *Shh* expression in hair buds (20, 21) and is repressed in SwG buds (Fig. 4A and fig. S3A). Additionally, *ENGRAILED* has been previously reported to act directly to transcriptionally repress *Shh* and/or the genes encoding its downstream transcriptional effectors (31–34). All of these pathways appeared to converge on repression of *Shh* and SHH signaling in SwG-permissive skin.

Regional BMP:SHH antagonism dictates SwG versus HF fates

To evaluate whether *Shh* up-regulation in ventral foot skin epithelium can prevent SwG fate, we

again turned to the inducible lentiviral system. Analogous to our experimental design for *Noggin*, we induced *Shh* for several days at E16.5. By P0, foot skin epithelial buds ectopically induced LHX2 and other HF markers and failed to progress to SwGs (Fig. 6D and fig. S6E, left), whereas in the underlying mesenchyme, signs of SHH signaling were seen, including a rise in BMP inhibitors (fig. S6E, right). Similar to our findings with *Noggin* induction, when *Shh* was induced after sweat buds were already established, gland formation progressed, as evidenced by morphology, luminal marker K18, and SwG markers NKA and AQUAPORIN-5 (Fig. 6E and fig. S6F). This was true even after 10 days of a high level of *Shh* expression (Fig. 6E), suggesting that once the narrow window of permissiveness is passed, glandular fate is no longer sensitive to SHH.

Last, we tested whether ectopic SHH administered during the permissive window has long-term consequences on appendage choice. We activated epithelial *Shh* in embryos for only 2 days at the sweat bud-formation stage and then stopped Doxy treatment and examined the foot pads of adult mice ~3 months later. Although morphology was clearly abnormal, the activation of hair-specific markers and absence of gland-specific markers provided compelling evidence that brief exposure to SHH during the critical window of gland bud morphogenesis was sufficient to cause irreversible changes in developing buds, preventing SwG fate and promoting features typically associated with HF fate (Fig. 6F). Additionally, when we generated mouse embryos whose skin contained mosaic patches of a constitutively active SHH receptor (SmoM2) (35) in the epithelium, only wild-type foot skin patches displayed K18⁺ SwGs, whereas SmoM2 patches expressed LHX2⁺ budlike structures (fig. S7). This suggested that selective activation of SHH signaling within the ventral foot skin epithelium is sufficient to trigger the fate switch, regardless of dermal signaling feedback.

Temporal regulation of BMP:SHH antagonism endows human skin with both SwGs and HFs

Our data are summarized in the model shown in Fig. 7A. We tested this model by addressing whether it can be used to explain how human skin is able to accommodate both SwGs and HFs within a common dermis. The organized patterning of glands and HFs within human skin argues against the notion that its dermis simply maintains intermediate levels of FGFs and BMPs, permissive for both types of epidermal appendages. Rather, our mouse data predict that within embryonic human dermis, there must be a temporal switch in dermal permissiveness, which allows for the sequential emergence of each appendage type. Indeed, upon analyzing human fetal scalp skin at gestational ages 15, 17, and 20 weeks with our markers established from mouse studies, we observed temporal segregation of waves of HF and SwG formation, with hair bud morphogenesis beginning to phase out between 15 and 17 weeks and sweat buds

emerging at 17 weeks (Fig. 7, B and C). By 20 weeks, sweat buds were still forming, in the absence of further signs of de novo HF morphogenesis.

To test whether a temporal change in BMP, FGF, and SHH signaling drives the intriguing developmental switch from hair to sweat fate within human scalp skin, we FACS-purified epithelial bud and mesenchymal cells (fig. S8, A and B) and analyzed their relative gene expression at these key windows of development. Within scalp dermis, expression of *BMP2*, *BMP4*, *BMP5*, *FGF2*, and *FGF7* were all significantly up-regulated at week 17 relative to week 15, which is coincident with the shift from hair to sweat bud formation (Fig. 7D). Thus, in contrast to mice, in which these dermal BMPs and FGFs were regionally restricted to the ventral foot skin, in humans the same genes were now temporally restricted to establish discrete hair versus sweat bud windows of permissiveness during embryogenesis of dorsal scalp skin.

To determine whether humans maintained evolutionary vestiges of regional skin differences, we also compared human scalp and palm skins at these gestational stages. As shown in Fig. 7E, the sweat bud-promoting mesenchymal signals that were elevated temporally by week 17 in human scalp skin were already present by week 15 in human palm skin, which was exclusive for SwG appendages (fig. S8C). Like in mouse foot skin, *BMP5* was the most prominent BMP expressed in palmar dermis (Fig. 7E), and LHX2 was exclusively expressed in palmar dermis and scalp HF epithelium (fig. S8C).

Consistent with the antagonistic regulatory circuitry that we unveiled in mouse, *SHH* expression decreased dramatically within the scalp epithelial buds as mesenchymal BMPs and FGFs, and epithelial *ENGRAILED-1*, were elevated (Fig. 7F). Additional signs of the developmental switch in appendage specification were revealed by temporal up-regulation of BMP target gene *ID1* and SwG marker *NKA* (Na/K-ATPase) (Fig. 7F). The sequential appearance of the appendages was similar but temporally delayed in dorsal limb skin, which like scalp is typified by both hair and SwGs (fig. S8D). In contrast in human palm skin, which (like its mouse counterpart) develops exclusively SwGs, *SHH* was already markedly suppressed regionally by week 15, which is coincident with the low level of WNT-(LEF1)-signaling and high level of *ENGRAILED-1* at this early time (Fig. 7G). Overall, our findings underscore the distinct temporal flip in BMP:SHH signaling that occurs in human development and endows human skin with the ability to permit sweat bud fate commitment as the final wave(s) of bud development in otherwise hairy skin.

Discussion

In the evolutionary developmental biology view of BMP signaling and fate specification of integument, both chicken scales (36) and mouse and human SwGs (our study) require BMP signaling to specify their fate, whereas feathers (37) and HFs (13) must suppress it. Thus, the differential

impact of BMP signaling on appendage fate specification has ancient roots and occurs repeatedly throughout vertebrate evolution in making epidermal bud fate decisions. In addition to its role in ectodermal fate specification, BMP-SHH antagonism is also critical for the patterning of chicken endodermal digestive epithelia (38, 39), especially in the glandular region of the stomach. Together with other findings on BMP requirement for various ectodermal glands (mammary and meibomian) (40, 41), a particular role begins to emerge for BMP signaling in promoting glandular fate, typically occurring on the ventral side of the body. Our studies provide insights into how elevated mesenchymal BMP signaling drives a thereafter self-perpetuating molecular cascade that culminates in silencing SHH signaling to suppress one appendage fate and specify another. By spatially regulating BMP-SHH antagonism, mammals can specify HF and glandular fates in distinct body sites. With additional temporal regulation of BMP-SHH antagonism, human skin has the special capacity first to generate waves of HF fates and then to switch midstream to specify glandular fates. Our findings pave the way for future therapeutic advances in skin regeneration with dual epithelial appendages.

Materials and methods

Mice and in utero lentiviral injection

All animals used for the experiments in this manuscript were generated previously: *K14Cre* (Fuchs lab) (42), *Bmpr1a^{fl/fl}* (kindly from Y. Mishina) (43), *SEA/GnJ* (The Jackson Laboratory) (44), *K14-rtTA* (Fuchs lab) (45), *Smo^{fl/fl}* (The Jackson Laboratory) (46), *R26SmoM2* (The Jackson Laboratory) (47), *R26^{Flox-Stop-Flox-YFP}* (The Jackson Laboratory) (48), *Ctrnbl^{fl/fl}* (kindly from R. Kemler) (49), *Axin2-LacZ* (The Jackson Laboratory) (50). *In utero* ultrasound-guided lentiviral injection procedures and preparation of high-titer lentivirus have been described (12). *K14rtTA* transgenic male (CD1 strain) were mated with CD1 wild type female, and their embryos were injected with high-titer lentivirus at E9.5. Each lentiviral injection experiments were performed at least two times and all foot skins from 2–4 animals were collected and analyzed at each time points. Animals were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-accredited animal facility, and procedures were performed with Institutional Animal Care and Use Committee (IACUC)-approved protocols. *rtTA* transcription factor was activated by feeding mice with doxycycline (2 mg/kg) chow at times specified. Lentiviral doxycycline-inducible *Shh* expression construct (*pLKO-TRE-Shh-PGK-H2BGFP*) has been previously described (30). Construct for the lentiviral Cre transductions has been previously described (12). Mouse *Noggin* was cloned by PCR from pMgB950 (kindly from R. Harland) to replace *Shh* in the *pLKO-TRE-Shh-PGK-H2BGFP* to generate *pLKO-TRE-Nog-PGK-H2BGFP*. Mouse *Bmp5* and *Fgf18* cDNAs were purchased from Sino Biological Inc. and were cloned into *pLKO-TRE-PGK-H2BGFP* vector.

Human tissue procurement

Fetal skin samples (scalp and palm) of 15, 17, 20 wks (2–3 samples for each stage) were procured by Novogenix Laboratories (Los Angeles, CA) and Advanced Bioscience Resources (Alameda, CA) in compliance with federal and state laws, and National Institute of Health guidelines. Tissues were processed and paraffin-embedded by HistoWiz, Inc. Standard histology and Alkaline Phosphatase immunohistochemistry staining were also performed at HistoWiz (Brooklyn, NY).

Quantification of sweat ducts

Experimental details have been described previously (3). Briefly, mouse hind foot skins were removed by dissection and incubated in EDTA (50 mM) 37°C for 30 min to separate epidermis and dermis. Under these conditions, sweat ducts remain associated with the epidermal cell sheet. Whole mount histochemical staining was with 0/1% Nile Blue A (Sigma). After washing, sweat ducts showed intense blue staining and were scored and imaged using a Leica M165 FC stereomicroscope.

In situ hybridization

In situ hybridization for *Shh* was performed as described previously (19).

Immunofluorescence and imaging

Skin (dorsal back and ventral foot) samples from 3–4 animals for each experiment were dissected and embedded in OCT (Tissue Tek) and cryo-sectioned at a thickness of 10–12 μ m. Tissue sections were fixed in 4% PFA and then blocked in gelatin block (1% gelatin, 2.5% normal donkey serum, 1% BSA, 0.3% Triton in PBS) 1 hour at RT. Primary antibodies were incubated at 4°C overnight. After washing with PBS, samples were incubated for 2 hours at room temperature with secondary antibodies conjugated with Alexa488, RRX, or Alexa647 (1:800, 1:400, and 1:400, respectively, Life Technologies) and 4'6-diamidino-2-phenylindole (DAPI). Samples were washed again with PBS and then mounted in Prolong Gold (Invitrogen). EdU incorporation was detected by Click-It EdU AlexaFluor594 Imaging Kit (Invitrogen) per manufacturer's instructions. The following primary antibodies were used: P-Cadherin (Rat, 1:50, Fuchs lab), E-Cadherin (Rat, 1:100, Fuchs lab), phospho-Smad1/5/8 (rabbit, 1:800, Cell signaling), Lef1 (rabbit, 1:300, Fuchs Lab), K5 (rabbit, 1:600, Fuchs Lab), K5 (guinea pig, 1:500, Fuchs Lab), Ki7 (rabbit, 1:600, Fuchs Lab), Ki18 (rabbit, 1:400, Fuchs Lab), LHX2 (rabbit, 1:2000, Fuchs Lab), anti-GFP/YFP (chicken, 1:2000, Abcam), ITGa6 (Rat, 1:100, BD), ITGb4 (Rat, 1:100, BD), Alkaline phosphatase (Goat, 1:100, R&D), β -Galactosidase (chicken, 1:2000, Abcam), NKA (Rabbit, 1:8000, Abcam), AQP5 (rabbit, 1:2000, Abcam), EpCam-Alexa488 (mouse, 1:100, Cell signaling). Epifluorescence images were acquired with an AxioObserver.Z1 microscope equipped with a Hamamatsu ORCA-ER camera (Hamamatsu Photonics), and with an ApoTome.2 (Carl Zeiss) slider that reduces the light scatter in the flu-

orescent samples. 20 $\times$ and 40 $\times$ objectives were used. Data collection was controlled by Zen software (Carl Zeiss).

Tissue preparation and fluorescence-activated cell sorting

Mouse E14.5 dorsal back skin and E17.5 ventral foot skin of *Axin2-LacZ* reporter mice were dissected and incubated in EDTA (50 mM), 37°C for 30 min to allow separation of epidermis and dermis. Note that for collecting E17.5 foot skin, only front feet were used, to avoid potential contamination of HF-competent dermal cells in the inter-pad region of the hind feet. Since these are skin tissues harvested at the placode formation stage, epidermal placodes were mostly removed with epidermal fraction and minimum epidermal cells remained with dermal fraction. The dermal tissues were then digested in Hanks Balanced Salt Solution (Lonza) with 1000 U/ml collagenase and 300 U/ml hyaluronadase (Sigma) and washed and re-suspended in PBS with 4% FBS to make single cell suspension. The following antibodies were used for fluorescence-activated cell sorting (FACS): α 6-PE (CD49f, 1:1000, eBioscience), CD140a-APC (1:100, eBioscience). Staining of intracellular β -galactosidase was performed using The Fluoreporter *lacZ* Flow Cytometry Kit (Molecular Probes) as per manufacturer's instructions. DAPI was used to exclude dead cells. Cell isolations were performed on FACSaria sorters equipped with DIVA software (BD Biosciences).

Human skin tissues were digested in LiberaseTL (Roche), 37°C for 1 hour 20 min. Digested tissues were resuspended and washed in PBS with 4% FBS to make single cell suspensions. The following antibodies were used for FACS: CD140a-PE (1:50, BioLegend), EpCAM-Alexa488 (1:100, Cell signaling), CD49f-PECy7 (1:1000, eBioscience), CD133-APC (1:100, Miltenyi). DAPI was used to exclude dead cells. Cell isolations were performed on FACSaria sorters equipped with DIVA software (BD Biosciences).

Explant culture and RNA extraction and RT-qPCR

Six to eight pieces of E17.5 foot skin from >3 different mouse embryos per condition were placed as explants into culture medium. The culture conditions were modified from the hanging-drop method (51), and 100–120 μ l of culture medium were used for each drop. The final concentrations of recombinant proteins and inhibitors used were: bFGF (50 ng/ml), FGF7 (50 ng/ml), FGF18 (50 ng/ml), BMP4 (100 ng/ml), BMP5 (100 ng/ml), Noggin (4 μ g/ml), MEKi (U0126, 10 μ M), MAPKi (SB203580, 1 μ M). After overnight culture (16–18 hour), skin tissues were incubated in EDTA (50 mM), 37°C for 20 min, and epidermis and dermis were separated and immediately frozen in liquid nitrogen. For embryonic foot skin tissues from lentiviral injection experiments to be analyzed by RT-qPCR, note that only front foot skins were collected because of higher infection efficiency compared to hind foot skin, and at least 6–8 pieces from 3–4 embryos were collected for each experiment.

For embryonic dorsal skin from lentiviral injection experiments, samples from head region (highest infection efficiency) were collected from at least two different embryos. All these samples were similarly incubated in EDTA (50 mM), 37°C for 20 min, to separate epidermis/dermis and were immediately frozen. The frozen tissues were homogenized using Bessman Tissue Pulverizer (Spectrum™) and collected in Trizol (Invitrogen). RNA was extracted with a Direct-zol RNA MiniPrep kit (Zymo Research) as per manufacturer's instructions. Equivalent amounts of RNA were reverse-transcribed by SuperScript VILO cDNA Synthesis Kit (Invitrogen). cDNAs were mixed with indicated primers and Power SYBR Green PCR Master Mix (Applied Biosystems), and quantitative PCR (qPCR) was performed on a Applied Biosystems 7900HT Fast Real-Time PCR system. cDNAs were normalized to equal amounts using primers against *Hprt* or *Ppib*. Primer sequences for RT-PCR were obtained using NCBI Primer-BLAST webtool.

RNA-seq analyses and statistics

For RNA-seq, RNA quality was determined using Agilent 2100 Bioanalyzer. Samples used for sequencing had RNA integrity numbers (RIN) > 8. Library preparation using Illumina TrueSeq mRNA sample preparation kit was performed at the Weill Cornell Medical College Genomic Core facility, and RNAs were sequenced on Illumina HiSeq 2000 machines. RNA reads were aligned to the mm9 build of the mouse genome using Tophat. Transcript assembly and differential expression was done using Cufflinks with Refseq mRNAs to guide assembly (52). Analysis of RNA-seq data was performed using the cummeRbund package in R (53). DAVID bioinformatic database was used to find enriched functional GO-term annotations in RNA-seq data (<https://david.ncifcrf.gov/content.jsp?file=citation.htm>). Box-and-whisker plots were generated using Prism5 software (GraphPad) to determine the significance between two groups. Error bars plotted on graphs denote SD. Unpaired student's *t* test was used to determine the significance between two groups: **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; ns, not significant.

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SUPPLEMENTARY MATERIALS

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RESEARCH ARTICLES

STRUCTURAL BIOLOGY

A three-dimensional movie of structural changes in bacteriorhodopsin

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Bacteriorhodopsin (bR) is a light-driven proton pump and a model membrane transport protein. We used time-resolved serial femtosecond crystallography at an x-ray free electron laser to visualize conformational changes in bR from nanoseconds to milliseconds following photoactivation. An initially twisted retinal chromophore displaces a conserved tryptophan residue of transmembrane helix F on the cytoplasmic side of the protein while dislodging a key water molecule on the extracellular side. The resulting cascade of structural changes throughout the protein shows how motions are choreographed as bR transports protons uphill against a transmembrane concentration gradient.

Energy-coupled membrane transport proteins are ubiquitous in biology. The basic framework underpinning unidirectional membrane transport is called the alternating access model and was proposed by Jardetzky half a century ago (*1*). This theory postulates that a high-affinity binding site, which is initially accessible to one side of the membrane, is converted through structural changes into a low-affinity binding site that is accessible to the other side of the membrane (fig. S1). The recent advent of time-resolved serial femtosecond crystallography (TR-SFX) at an x-ray free electron laser (XFEL) (*2–4*) provides an opportunity to examine this framework.

Bacteriorhodopsin (bR) harvests the energy content of light to drive conformational changes leading to unidirectional proton transport. Energy stored within a transmembrane proton concentration gradient is converted by adenosine triphosphate (ATP) synthase into ATP or is coupled to other transport processes. Considerable effort

has been made to understand how structural changes in bR transport a proton uphill against a transmembrane potential (*5–7*). Crystallographic structures derived from data recorded at cryogenic temperature reveal both light- and mutation-induced structural changes in bR (*6, 7*), but this work is controversial for several reasons: (i) The reported structures show considerable variation (*7*), (ii) structures trapped at cryogenic temperature or by mutation do not correlate directly with time-dependent structural changes in wild-type bR, and (iii) x-ray induced radiation damage may mislead the interpretation of trapped intermediate structures (*8–10*). We circumvented these concerns by using TR-SFX to record a three-dimensional (3D) movie of structural changes in bR at room temperature at 2.1-  resolution (*2–4*). We collected our data by using 10-fs-long XFEL pulses at the SPring-8 Angstrom Compact Free Electron Laser (SACLA). The principle of “diffraction before destruction” (*11*) ensures that these crystallographic structures are effectively

free from the influence of x-ray-induced radiation damage (*12*).

Structure of the bR resting conformation

Bacteriorhodopsin is a seven-transmembrane α -helix protein containing a buried all-trans retinal chromophore that is covalently attached, through a protonated Schiff base (SB), to Lys²¹⁶ of helix G. Light photoexcites the chromophore, which isomerizes with high quantum yield to a 13-cis configuration (Fig. 1A) and thereby initiates a sequence of spectral (fig. S2) and structural changes that facilitate spontaneous proton exchange between acidic and basic amino acid residues (Fig. 1B). Electron density for the resting-state bR structure (Fig. 1C; see also table S1 for crystallographic data) shows how the protonated SB forms a hydrogen-bond (H-bond) interaction with a water molecule, labeled Wat402 (*13*). This interaction is critical for attaining the high SB proton affinity with a pK_a of 13.3 (where K_a is the acid dissociation constant) (*14*). Asp⁸⁵, the primary proton acceptor, also forms an H-bond interaction with Wat402, and additional H-bond interactions with Thr⁸⁹ and Wat401 ensure its low resting-state pK_a of 2.2 (*15*). A difference of 11 orders of magnitude between the proton affinities of the primary donor and acceptor prevents the leakage of protons from the extracellular (EC) medium to the cytoplasm (CP) but raises the question of what brings these proton affinities close together to facilitate spontaneous proton exchange? It is also puzzling why it takes microseconds for the primary proton transfer to occur when the SB and Asp⁸⁵ are initially separated by only 4   and a water-mediated proton exchange pathway between these two groups is seen in the resting state (*13*). Moreover, protons are pumped from the CP to the EC, yet retinal isomerization redirects the SB proton away from the EC and toward the CP (Fig. 1A), which appears to contradict Jardetzky's framework.

Time-resolved spectroscopy studies of bR microcrystals

Before returning to the bR resting state, photoactivated bR progresses through a sequence of spectral intermediates labeled K, L, M₁ (early M), M₂ (late M), N, and O (fig. S2). A proton is transferred from the SB to Asp⁸⁵ in the L-to-M transition and from Asp⁹⁶ to the SB from the M-to-N transition. Time-resolved difference absorption spectra from bR microcrystals suspended in a lipidic cubic phase matrix (fig. S3A) show a photocycle turnover similar to that of

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wild-type bR in the purple membrane. Spectral decomposition reveals that for a time delay $\Delta t \leq 10$ μ s, the photoactivated population in microcrystals is dominated by the L intermediate but with traces of K, whereas the M-intermediate population increases to 50% at $\Delta t = 19$ μ s and falls to 50% at $\Delta t = 10$ ms (fig. S3, B and C). Measurements from four independent crystal batches yield the half-rise for the M-state as 15 ± 7 μ s and the half-decay as 8 ± 3 ms.

Overview of structural rearrangements within the bR photocycle

In TR-SFX, a continuous stream of microcrystals is injected across a focused XFEL beam, and the delay between sample photoactivation and the arrival of an XFEL pulse is controlled electronically (2–4) (fig. S4). TR-SFX data were recorded to 2.1-Å resolution from light-adapted bR microcrystals after photoactivation by a nanosecond laser pulse for $\Delta t = 16$ ns, 40 ns, 110 ns, 290 ns, 760 ns, 2 μ s, 5.25 μ s, 13.8 μ s, 36.2 μ s, 95.2 μ s, 250 μ s, 657 μ s, and 1.725 ms (table S1), which are evenly spaced on a logarithmic scale. A global overview of the evolution of difference electron density reveals how structural changes first emerge near the active site of the protein and become stronger around Lys²¹⁶ of helix G and Trp¹⁸² of helix F before cascading toward the EC side of the protein along helix C (fig. S5 and movie S1).

Early structural changes in the bR photocycle

For $\Delta t = 16$ ns, which corresponds to essentially pure K intermediate, paired negative and positive difference electron density features adjacent to the C20 methyl group reveal that the retinal is initially tilted toward helix G in response to photoisomerization (Fig. 2A). Combined quantum mechanics and molecular mechanics computations also favor a twisted retinal geometry (fig. S6) and indicate that ~ 17 kcal/mol of energy is initially stored within this distorted active site configuration, which equates to 32% of the absorbed photon's energy and is compatible with earlier estimates (16, 17). By $\Delta t = 290$ ns, the positive difference density feature associated with the C20 methyl is much weaker and moves into the plane of the retinal, whereas paired negative and positive difference electron density features associated with Trp¹⁸² grow stronger (Fig. 2B), showing how Trp¹⁸² is displaced toward the CP as the retinal straightens (Fig. 2C). Negative and positive difference electron density features also indicate that a movement of the side chain and backbone of Lys²¹⁶ has begun at $\Delta t = 16$ ns (movies S2 and S3).

A strong negative difference electron density peak visible at $\Delta t = 16$ ns on Wat402 (Fig. 2A) shows that this water molecule is rapidly disordered by retinal isomerization. This feature doubles in strength as the photocycle evolves (Fig. 3A, movies S2 and S3, and table S2), indicating that the freedom of Wat402 to move is initially constrained by the limited size of the cavity in which it is buried. However, this water becomes increasingly mobile as the retinal is

displaced toward the CP. Low-temperature trapping studies have suggested that Wat402 disorders upon retinal photoisomerization (18), but this conclusion was challenged in light of similar observations due to x-ray induced radiation damage (8–10). Because no effects of radiation damage are visible when using ≤ 10 -fs XFEL pulses at an x-ray dose of 12 MGy (12, 19), these TR-SFX data bring closure to this debate. It is noteworthy that one trapped K-intermediate showed Wat402 disordering (18) but did not visualize a twist of the retinal C20 methyl toward helix G (Fig. 2, A and C), whereas another captured this twist but kept Wat402 in the K-state model (8), and a third K-state structure showed neither change (20).

Conformational changes associated with proton transfer

The key step in achieving unidirectional proton transport by bR is the primary transfer event from the SB to Asp⁸⁵ (the L-to-M transition) because only the mutation of Asp⁸⁵ (21) or the removal of retinal completely stops proton pumping. Our data reveal a smooth evolution of elec-

tron density changes on the CP side of helices F and G before proton transfer (Fig. 3B and movies S1 and S3). Paired positive and negative density features also show that the side chain of Leu⁹³ is displaced toward the CP and a weaker positive difference electron density peak arises between the retinal, Leu⁹³, and Thr⁸⁹ from $\Delta t = 40$ ns until 13.8 μ s (Fig. 4A) and is strongest at $\Delta t = 760$ ns (Fig. 3C and table S2). We modeled this feature as a transient water molecule [Wat452 (W452) in Fig. 4B] that shows weak H-bond interactions with the SB (3.35 Å) and the backbone carbonyl oxygen of Thr⁸⁹ (3.35 Å). Its crystallographic distance to O γ of Thr⁸⁹ of 3.95 Å may also be classified as a weak H-bond interaction (22). Structural refinement shows that for $\Delta t = 16$ ns, O γ of Thr⁸⁹ is separated from the SB by 3.97 Å and the SB nitrogen is pointing toward helix G, whereas for $\Delta t = 760$ ns the SB nitrogen rotates almost 180° toward helix C and reduces the SB-O γ separation to 3.30 Å. Fluctuations about these crystallographic positions could therefore create a transient proton-transport pathway linking the SB to Asp⁸⁵ through Wat452 and Thr⁸⁹ (Fig. 4B).

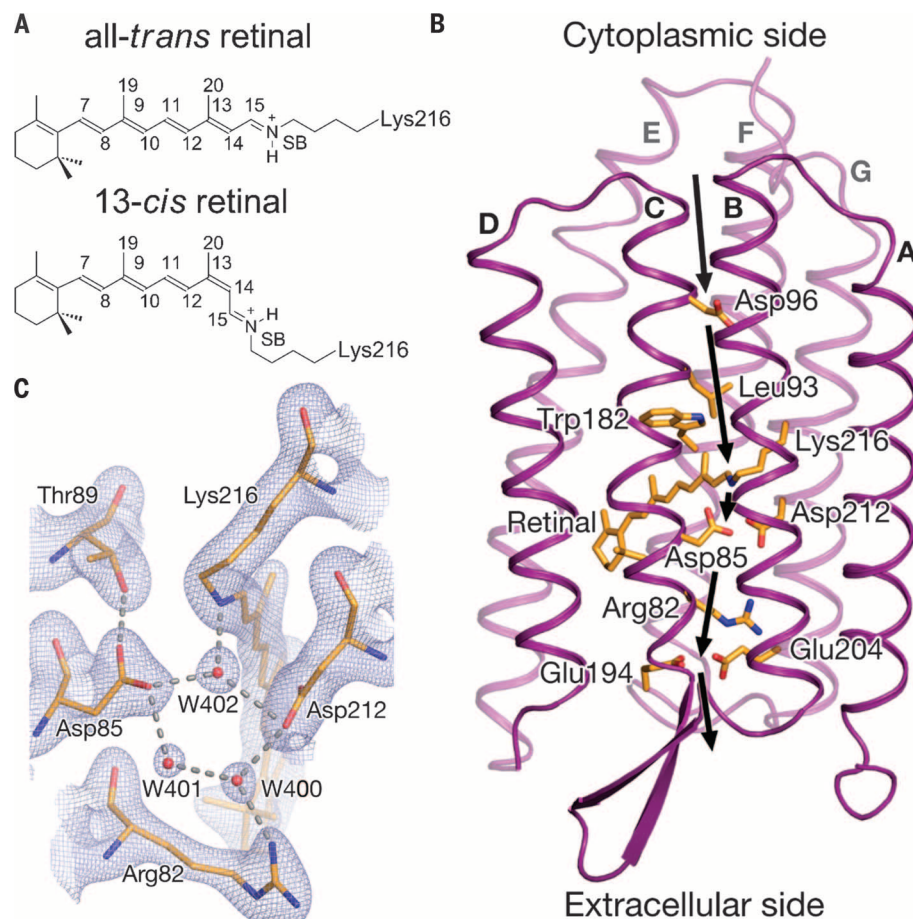


Fig. 1. Structure and function of bacteriorhodopsin (bR). (A) Schematic illustrating retinal covalently bound to Lys²¹⁶ through a protonated Schiff base (SB) in an all-trans and a 13-cis configuration. (B) Proton-exchange steps (arrows) achieving proton pumping by bR. The primary proton-transfer step is from the SB to Asp⁸⁵ and corresponds to the spectroscopic L-to-M transition. (C) $2mF_o - DF_{calc}$ electron density for the bR active site in its resting conformation. Electron density (gray) is contoured at 1.3σ (σ is the root mean square electron density of the map). W400, W401, and W402 denote water molecules.

Fig. 2. Early structural changes in the bR photocycle.

(A) View of the $|F_{\text{obs}}|_{\text{light}} - |F_{\text{obs}}|_{\text{dark}}$ difference Fourier electron density map near the retinal for $\Delta t = 16$ ns.

Blue indicates positive difference electron density; yellow denotes negative difference electron density (contoured at $\pm 3.7\sigma$). The resting-state bR model

(purple) was used for phases when calculating this map. Paired negative and positive difference electron densities indicate a sideways movement of the retinal's C20 methyl. (B) Identical representation but for the time point $\Delta t = 290$ ns. (C) Crystallographic models deriving from partial-occupancy refinement for $\Delta t = 16$ ns (blue) and $\Delta t = 290$ ns (red) superimposed upon the resting bR structure (purple, partially transparent).

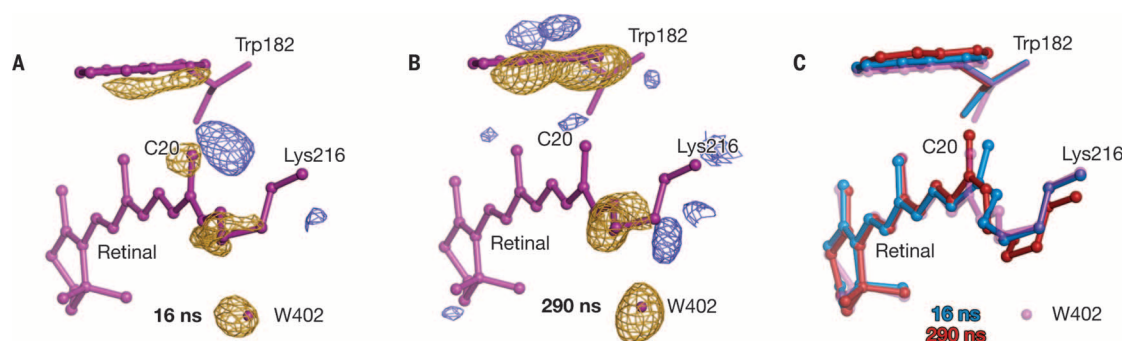
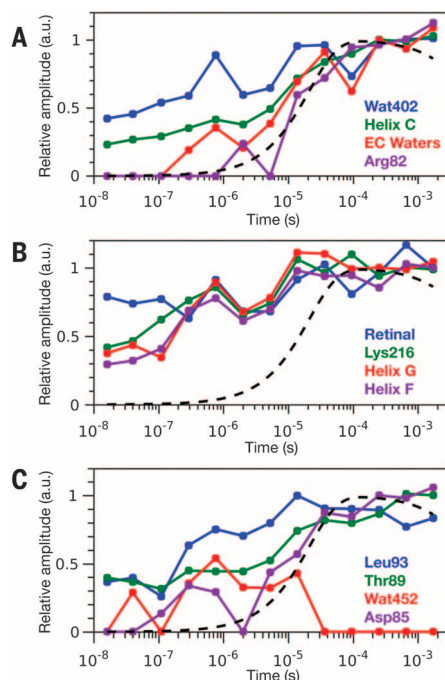


Fig. 3. Relative changes of electron density amplitudes in bR with time.

(A) Time-dependent root mean square difference electron density amplitudes associated with Wat402 (blue); helix C (green, backbone of Arg⁸², Ala⁸⁴, and Asp⁸⁵); waters 400, 401, and 451 (red, EC waters); and the side chain of Arg⁸² (purple). a.u., arbitrary units. (B) Time-dependent amplitudes associated with the retinal (blue), the side chain of Lys²¹⁶ (green), helix G (red, backbone of Ser²¹⁴, Ala²¹⁵, and Lys²¹⁶) and helix F (purple, side chains of Trp¹⁷⁸ and Trp¹⁸² and backbone of Arg¹⁷⁵). (C) Time-dependent amplitudes associated with Leu⁹³ (blue), the side chain of Thr⁸⁹ (green), Wat452 (red), and the side chain of Asp⁸⁵ (purple). Amplitudes were averaged over the last four time points and scaled to 1 [except Leu⁹³ and Wat452 (42)]. Amplitudes for $\Delta t = 95.2 \mu\text{s}$, $657 \mu\text{s}$, and 1.725 ms are scaled by 2.0, 1.5, and 0.8, respectively (42). Linear decomposition of electron density amplitudes shows how these motions are coordinated (fig. S8). The M-state population measured from microcrystalline slurries is indicated for comparison (dashed black line).



A water molecule was built at the Wat452 location in one cryo-trapped L-intermediate structure (23) but was not observed in any other L-state study (24–26). Fourier transform infrared (FTIR) spectroscopy observations have inferred that a transient H-bond interaction of the SB to a water molecule arises in the L-state (27, 28), and Wat452 is the only plausible candidate for this interaction. Computer simulations also establish that a water molecule at this position can channel a proton to Asp⁸⁵ through Thr⁸⁹ (29). Another transient water molecule (Wat453), which forms H-bond interactions with the carbonyl oxygen of Lys²¹⁶ and the backbone nitrogen of Gly²²⁰, was also predicted from FTIR spectroscopy studies (30) and is observed in the difference electron density.

On the EC side of the retinal, the disordering of Wat402 triggers a structural cascade visible from $\Delta t \geq 13.8 \mu\text{s}$ as significant ($\geq 4\sigma$) difference electron density peaks that show the disordering of Wat400 and Wat401 and the ordering of

a new water molecule, Wat451, between Asp⁸⁵ and Asp²¹² (Fig. 5 and table S2). Concomitant with these active-site water rearrangements is an increase in the strength of paired negative and positive difference density features along the EC portions of helix C (residues 82 to 89, Fig. 5B), which are modeled as a concerted inward movement of helix C toward helix G (Fig. 5C). These observations are similar to electron density changes recorded in low-temperature trapping studies of the bR L-state (7, 24, 25), but TR-SFX data show that this inward flex of helix C approaches its maximum amplitude as the SB is deprotonated [i.e., as the M-state population grows (Fig. 3A)]. Protons exchange between consecutive water molecules in the model channel gramicidin A on a subpicosecond time scale (31), and the primary proton transfer should be very fast once a structure facilitating this exchange is attained. Consequently, the time required for bR to evolve to a conformation with helix C bent toward helix G is the rate-limiting step that

controls the primary proton transfer and explains why it takes microseconds for the SB to be deprotonated.

Breaking the EC connectivity after proton transfer

A key conceptual issue underpinning membrane transport is the nature of the “switch,” or more specifically, how the SB breaks access to the EC side of bR after the primary proton transfer to Asp⁸⁵. This is central to the mechanism of proton pumping because structural changes must prevent Asp⁸⁵ from reprotonating the SB or the photocycle would be futile. A switch may be achieved by breaking the pathway for the reverse proton transfer or by perturbing the chemical environment of key groups to shift the equilibrium in favor of the forward proton-transfer reaction.

Difference electron density features associated with movements of the retinal, helix F (Trp¹⁸², Thr¹⁷⁸, and Arg¹⁷⁵), and helix G (Lys²¹⁶, Ala²¹⁵, and Ser²¹⁴) all show a stepwise increase in strength from $\Delta t = 5.25$ to $36.2 \mu\text{s}$, which coincides with the primary proton-transfer event (Fig. 3B). We therefore conclude that proton transfer from the SB to Asp⁸⁵ effects a structural change within the retinal, which we model as a displacement of the SB toward the CP by $0.15 \text{ \AA}$ and of the C20 methyl by $0.21 \text{ \AA}$, over the interval $760 \text{ ns} \leq \Delta t \leq 36.2 \mu\text{s}$. These changes may be due to the retinal being subtly straighter when deprotonated (32) or to proton transfer neutralizing the mutual electrostatic attraction between the SB and Asp⁸⁵ (6). Both geometric and electrostatic effects increase the separation between the SB and Asp⁸⁵ and would therefore increase the barrier for the reverse proton transfer.

Protein structural changes also hinder the reverse proton transfer from Asp⁸⁵ to the SB. Wat452, which may help mediate the primary proton transfer through Thr⁸⁹ (Fig. 4B), is observed for $40 \text{ ns} \leq \Delta t \leq 13.8 \mu\text{s}$ but is not visible after a proton is transferred to Asp⁸⁵ (Fig. 3C and table S2). An H-bond interaction connecting Thr⁸⁹ to Asp⁸⁵ is also lost after the primary proton-transfer event, with significant ($\geq 4\sigma$) negative difference electron density becoming visible between O γ of Thr⁸⁹ and O δ of Asp⁸⁵ for $\Delta t \geq 36.2 \mu\text{s}$ (Fig. 5D, movie S2, and table S2). These

structural changes break the reverse proton-transfer pathway from Asp⁸⁵ to the SB through Thr⁸⁹ and Wat452 while simultaneously increasing the pK_a of Asp⁸⁵. Thr⁸⁹ moves closer to the retinal in the M state with the H bond from Oy of Thr⁸⁹ to the SB decreasing from 3.30 Å at 760 ns to 3.04 Å at 36.2 μs and to 2.86 Å at 1.725 ms. This tighter H-bond donor interaction to the deprotonated SB locks Thr⁸⁹ in a conformation that allows the Asp⁸⁵ side chain to rotate and break its connectivity to Thr⁸⁹ while forming a new H-bond interaction with Wat451. In contrast with TR-SFX, FTIR spectroscopy studies at low temperature have predicted a tight Thr⁸⁹-Asp⁸⁵ H-bond interaction in the M state (33), but the complex dynamics of these H-bond interactions may preclude a unique interpretation of spectroscopic observations.

Synchronous with the M-state spectral transition is a displacement of the positively charged head group of Arg⁸² toward the EC (Figs. 3A and 5C), which implies that this movement is triggered by the protonation of Asp⁸⁵. This rearrangement displaces a positive charge away from Asp⁸⁵ and consequently increases the pK_a of the primary proton acceptor, hindering the reverse proton-transfer reaction (34). These structural perturbations also trigger a rearrangement of Glu¹⁹⁴ and Glu²⁰⁴, which were modeled from Δt ≥ 250 μs. Similar structural changes have been observed for trapped M intermediates and were argued to influence the release of a proton to the EC medium (34, 35). Overall, this sequence highlights structural changes that favor Asp⁸⁵ being protonated and reveal how the SB accessibility to the EC side of the protein is broken after the SB is deprotonated, thereby functionally coupling the primary proton-transfer event to the switch in the bR photocycle.

Structural changes on the CP side of bR

Correlated movements are observed on the CP side of helix F as paired negative and positive difference electron density stacking through Trp¹⁸², Thr¹⁷⁸, and Arg¹⁷⁵ (Fig. 6, A and B, and movie S3). As with electron density changes associated with the retinal and near the center of helix G, difference density features on CP portions of helix F arise rapidly but plateau once the SB is deprotonated (Fig. 3B). Structural refinement indicates that the retinal's C20 methyl group is displaced by 1.12 Å toward the CP over the time sequence sampled here, with a corresponding displacement of Trp¹⁸² of 0.96 Å. The largest Cα displacement is 1.13 Å and occurs for Lys²¹⁶ at Δt = 1.725 ms. In helix F, the Cα atoms of residues 174 to 178 are displaced by only 0.4 Å; therefore, as with cryo-trapped structures of M intermediates (34–37), TR-SFX does not reveal the large movements of helices E and F that were predicted from bR triple-mutant structures (32, 38). This discrepancy may be reconciled by noting that motions of these helices are severely restricted in the P6₃ crystal form because residues 165 and 166 of the E-F loop participate in crystal contacts with residues 232 and 234 of the C terminus (fig. S7). Although larger motions of the CP portions of

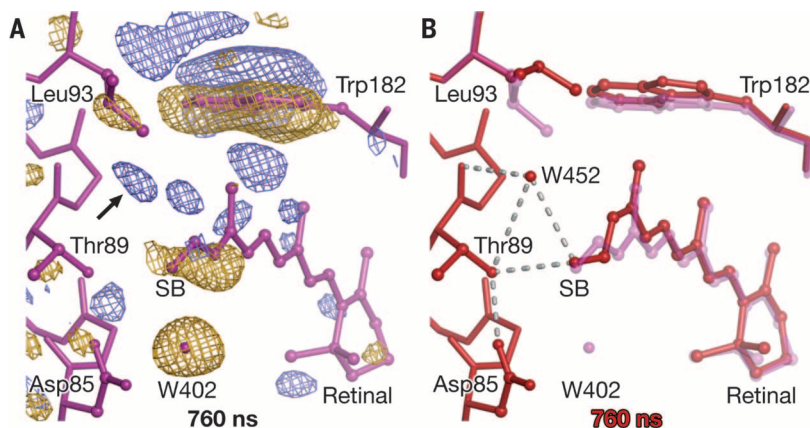


Fig. 4. Pathway for proton transfer from the SB to Asp⁸⁵. (A) View of the $|F_{\text{obs}}|^{\text{light}} - |F_{\text{obs}}|^{\text{dark}}$ difference Fourier electron density map (contoured at $\pm 3.1\sigma$) near the retinal for $\Delta t = 760$ ns. A positive difference feature (arrow) suggests the ordering of a water molecule (Wat452). (B) Crystallographic model for the time point $\Delta t = 760$ ns (red) superimposed upon the resting-state model (purple, partially transparent).

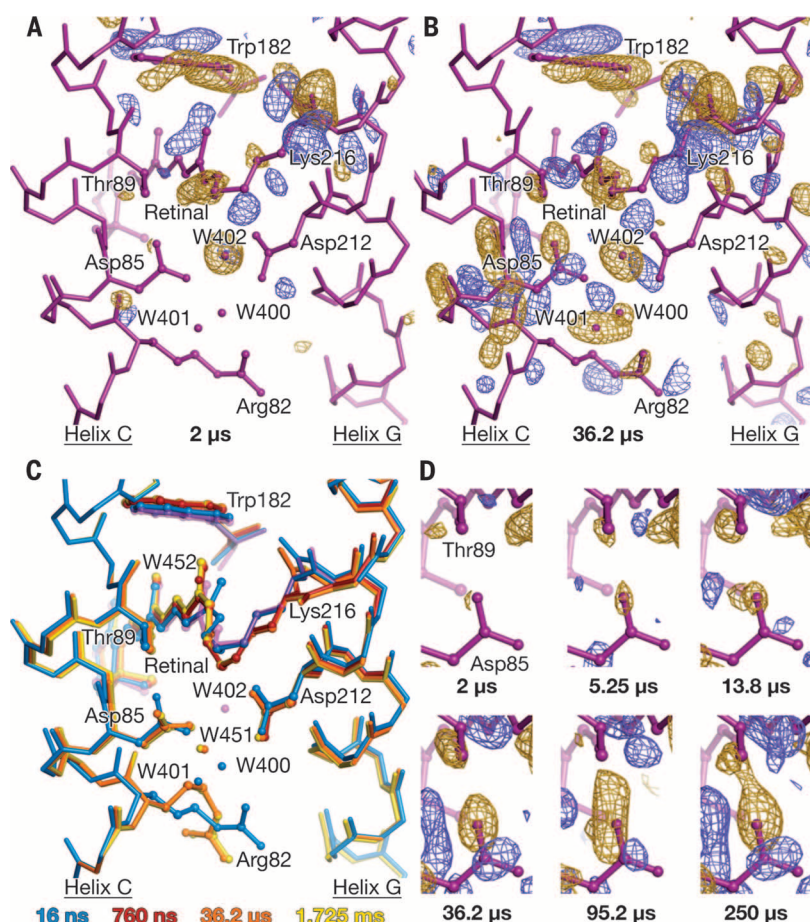


Fig. 5. bR conformation controlling the primary proton transfer event. (A and B) View of the difference Fourier electron density map immediately to the EC side of the retinal for (A) $\Delta t = 2$ μs and (B) $\Delta t = 36.2$ μs. All maps are contoured at $\pm 3.5\sigma$. Difference Fourier electron density maps from this viewpoint are shown for all 13 time points in movie S2. (C) Crystallographic structural models deriving from partial-occupancy refinement are superimposed upon the resting bR structure (purple, partially transparent) for $\Delta t = 16$ ns (blue), 760 ns (red), 36.2 μs (orange), and 1.725 ms (yellow). (D) Close-up view of difference electron density across the Asp⁸⁵-Thr⁸⁹ H bond for the time points $\Delta t = 2$ μs, 5.25 μs, 13.8 μs, 36.2 μs, 95.2 μs, and 250 μs. All maps are contoured at $\pm 3.5\sigma$ except for $\Delta t = 95.2$ μs, which is contoured at $\pm 2.8\sigma$.

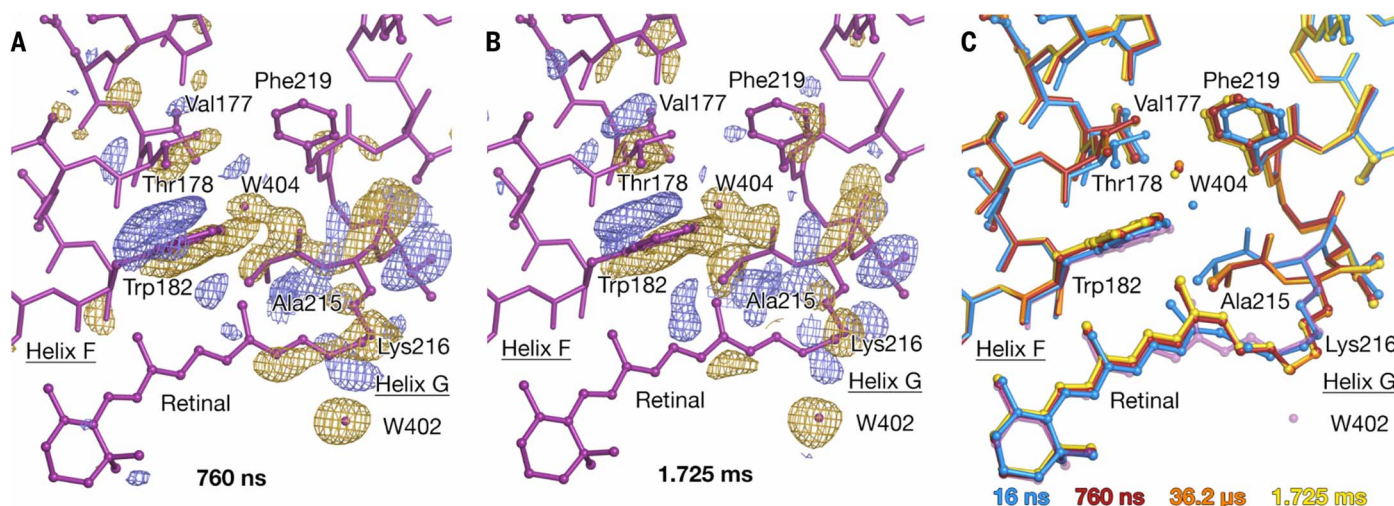


Fig. 6. Conformational changes on the CP side of bR. (A and B) Close-up view of the difference Fourier electron density map immediately to the CP side of the retinal for (A) $\Delta t = 760$ ns and (B) $\Delta t = 1.725$ ms. All maps are contoured at $\pm 3.5\sigma$. Difference Fourier electron density maps from this viewpoint are shown for all 13 time points in movie S3. (C) Crystallographic structural models deriving from partial-occupancy refinement are superimposed upon the resting bR structure (purple, partially transparent) for $\Delta t = 16$ ns (blue), 760 ns (red), 36.2 μ s (orange), and 1.725 ms (yellow).

helices E and F arise later in the bR photocycle (39, 40), these structural changes cannot be critical for the mechanism of proton pumping. This is because these motions are suppressed in 3D crystals yet the photocycle is similar to that of bR in the purple membrane (fig. S3), and the Asp⁹⁶→Gly⁹⁶/Phe¹⁷¹→Cys¹⁷¹/Phe²¹⁹→Leu²¹⁹ bR triple mutant is constitutively open to the CP yet is able to pump protons (41).

Mechanistic overview

Retinal isomerization reorients the SB proton into a hydrophobic cavity while breaking its H bond to Wat402, both of which lower the proton affinity of the SB (14). An initially twisted retinal becomes planar within 290 ns, causing Trp¹⁸² and Leu⁹³ to be displaced toward the cytoplasm and allowing a water molecule to order between Leu⁹³, Thr⁸⁹, and the SB in the L state. H-bond interactions from the protonated SB to Wat452 or Thr⁸⁹ create a pathway for proton transfer to Asp⁸⁵ (Fig. 4B) and explain how the SB makes contact with Asp⁸⁵ despite having been turned toward the CP by photoisomerization. A steric clash between C ϵ of Lys²¹⁶ and Wat402 dislodges this water molecule, triggering the collapse of the water-mediated H-bond network on the EC side of bR. This allows helix C to bend toward helix G approximately 10 μ s after photo-activation and raises the pK_a of Asp⁸⁵ to the point where it may spontaneously accept a proton from the SB. Once a proton is transferred, the Asp⁸⁵–Thr⁸⁹ H bond is lost (Fig. 5D), thus breaking the SB connectivity to the EC side of the protein. Consequently, diffraction data spanning five orders of magnitude in time reveal how structural changes in bR achieve unidirectional membrane transport half a century after Jardetzky first proposed the alternating access framework obeyed by all membrane transporters (1).

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P.N., M.S., C.S., D.N., R.D., Y.K., T.S., D.I., T.F., Y.Y., B.J., T.Ni., K.O., M.F., C.W., R.A., C.S., P.B., J.S., and R.N. performed data collection; M.K., T.Ki., and T.No. performed time-resolved visible absorption spectroscopy; C.W. and R.N. analyzed the spectral data; T.Na. and O.N. performed data processing; A.R. and E.N. refined the intermediate structures; A.-N.B. performed computations; K.T., C.S., T.Ka., T.Ha., Y.J., and M.Y. developed the SFX systems at SACLA; and R.N., C.W., E.N., A.R., M.K., and T.Na. wrote the paper with input from all authors. Coordinates and structure factors have been deposited in the Protein Data Bank with IDs 5B6V (bR resting state), 5B6W ($\Delta t = 16$ ns), 5H2H ($\Delta t = 40$ ns), 5H2I ($\Delta t = 110$ ns), 5H2J ($\Delta t = 290$ ns), 5B6X ($\Delta t = 760$ ns), 5H2K ($\Delta t = 2$ μ s), 5H2L ($\Delta t = 5.25$ μ s), 5H2M ($\Delta t = 13.8$ μ s), 5B6Y ($\Delta t = 36.2$ μ s), 5H2N ($\Delta t = 95.2$ μ s), 5H2O ($\Delta t = 250$ μ s), 5H2P ($\Delta t = 657$ μ s),

and 5B6Z ($\Delta t = 1.725$ ms). Raw diffraction images have been deposited in the Coherent X-ray Imaging Data Bank (accession ID 53).

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6319/1552/suppl/DC1
Materials and Methods
Figs. S1 to S9
Tables S1 and S2
References (43–76)
Movies S1 to S3

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TOPOLOGICAL MATTER

Majorana bound state in a coupled quantum-dot hybrid-nanowire system

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Hybrid nanowires combining semiconductor and superconductor materials appear well suited for the creation, detection, and control of Majorana bound states (MBSs). We demonstrate the emergence of MBSs from coalescing Andreev bound states (ABSs) in a hybrid InAs nanowire with epitaxial Al, using a quantum dot at the end of the nanowire as a spectrometer. Electrostatic gating tuned the nanowire density to a regime of one or a few ABSs. In an applied axial magnetic field, a topological phase emerges in which ABSs move to zero energy and remain there, forming MBSs. We observed hybridization of the MBS with the end-dot bound state, which is in agreement with a numerical model. The ABS/MBS spectra provide parameters that are useful for understanding topological superconductivity in this system.

As condensed-matter analogs of Majorana fermions—particles that are their own antiparticles (*1*)—Majorana bound states (MBSs) are anticipated to exhibit non-Abelian exchange statistics, providing a basis for naturally fault-tolerant topological quantum computing (*2–7*). In the past two decades, the list of potential realizations of MBSs has grown from even-denominator fractional quantum Hall states (*8*) and *p*-wave superconductors (*9*) to topological insulator-superconductor hybrid systems (*10*), semiconductor-superconductor (Sm-S) hybrid nanowire systems (*11–21*), and artificially engineered Kitaev chains (*22–24*). Sm-S hybrid systems have received particular attention because of ease of realization and a high degree of experimental control. Experimental signatures of MBS in Sm-S systems have been reported (*25–29*),

typically consisting of zero-bias conductance peaks in tunneling spectra appearing at finite magnetic field.

In a confined normal conductor-superconductor system, Andreev reflection will give rise to discrete electron-hole states below the superconducting gap—Andreev bound states (ABSs). Given the connection between superconducting proximity effect and ABSs, zero-energy MBSs in Sm-S hybrid nanowires can be understood as a robust merging of ABSs at zero energy, thanks in part to the presence of strong spin-orbit interaction (SOI) (*11–13, 15, 16*). However, not all zero-energy ABSs are MBSs. For instance, in the nontopological or trivial phase, ABSs can move to zero energy at a particular Zeeman field, giving rise to a zero-bias conductance peak, and then split again at higher fields, indicating a switch of fermion parity (*30*). On the other hand, zero-energy MBSs in short wires may also split as a function of chemical potential or Zeeman field (*14*). In this case, the difference between topological MBSs in a finite-length wire and trivial ABSs is whether the states are localized at the wire ends or not (*17*). We will use the term “MBSs” to refer to ABSs that are to a large degree localized at the wire ends and would evolve into true topological MBSs as the wire becomes longer. We also will use the term “topological phase in a finite-length wire”

to refer to the regime in which MBS appears. The similarities between trivial ABS zero-energy crossings and MBS in a finite-length wire can be subtle (*13, 15, 16, 30, 31*). Several obstacles have prevented a detailed experimental study of the ABS-MBS relation to date, including a soft proximity-induced gap (*18*), the difficulty of tuning the chemical potential of the hybrid nanowire, and disorder in the wire and tunneling barrier.

In this work, we investigated MBSs and their emergence from coalescing ABSs, using tunneling spectroscopy through quantum dots at the end of epitaxial hybrid Sm-S nanowires. We observed gate-controlled hybridization of the MBSs with the bound state in the end dot, finding excellent agreement between experiment and numerical models. The epitaxial Sm-S interface induces a hard superconducting gap (*32, 33*), whereas the partial coverage by the epitaxial superconductor allows tuning of the chemical potential and yields a high critical field (*34*), both crucial for realizing MBSs.

Hybrid nanowire with end dot

Our devices were made of epitaxial InAs/Al nanowires (Fig. 1A) (*32*). Wurtzite InAs nanowires were first grown to a length of 5 to 10 μ m by means of molecular beam epitaxy, followed by low-temperature epitaxial growth of Al. Two or three facets of the hexagonal InAs core were covered by Al (Fig. 1B) (*32*). The nanowires were then deposited onto a degenerately doped silicon/silicon oxide substrate. Transverse Al etch was used to selectively remove the Al from the end of the wire, which was then contacted by titanium/gold (Ti/Au, 5/100 nm), forming a normal (non-superconducting) metal lead. Five devices were investigated. Data from four devices, denoted 1 to 4, are reported in the main text, and data from a fifth device, denoted 5, are reported in (*35*). For device 1, the unetched end of the nanowire section was contacted by titanium/aluminum/vanadium (Ti/Al/V, 5/20/70 nm), and global back gate and local side gates were used to control the electron density in the wire. A quantum dot was formed in the 150-nm bare InAs wire segment between the Ti/Au normal contact and the epitaxial Al shell, owing to disorder or band-bending (*33*). Fabrication details for the other devices, each slightly different, are given in (*35*). Micrographs of all devices accompany transport data. Except where noted, the magnetic field *B* was applied parallel to the

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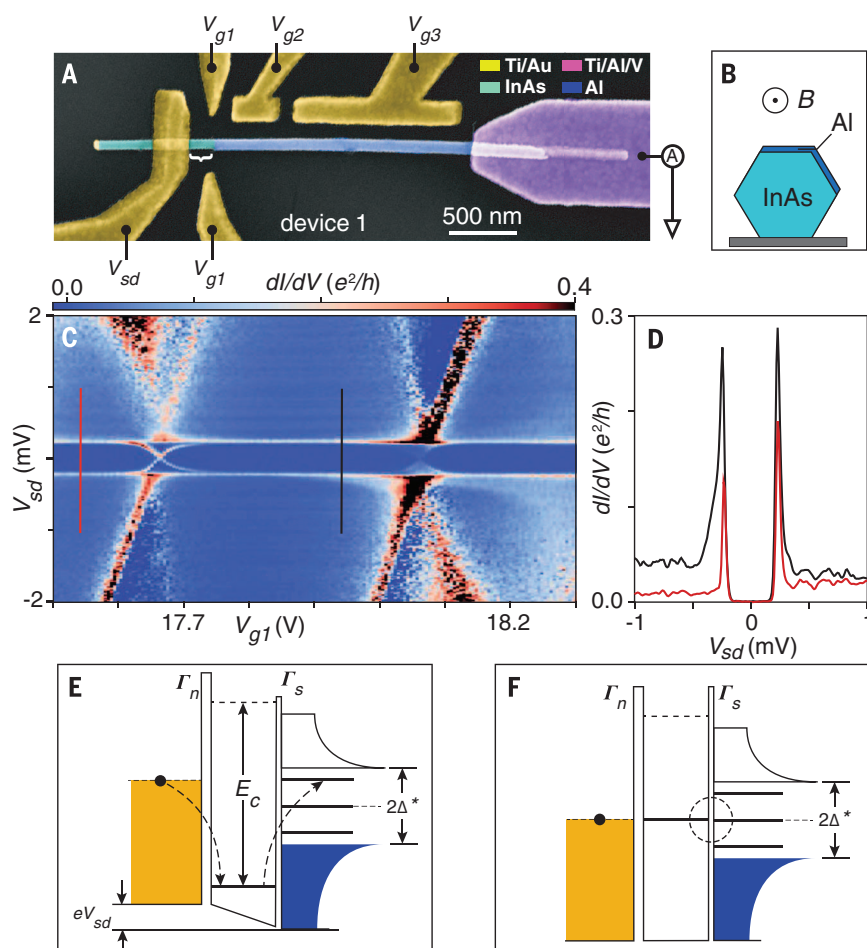


Fig. 1. Epitaxial hybrid nanowire with end dot. (A) Scanning electron micrograph (SEM) of device 1, with false color representing different materials. The white brace indicates the location of a natively formed quantum dot. (B) Schematic cross-sectional view of the nanowire. The epitaxial Al shell (dark blue) was grown on two facets of the hexagonal InAs core (light blue), with a thickness of ~ 10 nm. The applied magnetic field is parallel to the nanowire in most cases. (C) Differential conductance measured for device 1 as a function of applied source-drain bias voltage, V_{sd} , and the voltage V_{g1} on gate $g1$. A Coulomb diamond pattern and a low-conductance gap through the valleys can be seen. (D) Line-cuts of the conductance, taken from (C), indicated by red and black lines. (E and F) Schematic views of two different dot-wire configurations of the device. (E) illustrates the elastic cotunneling process in the Coulomb-blockade regime, whereas (F) shows how a quantum-dot level can hybridize with the subgap states in the nanowire when it is tuned to resonance.

nanowire axis by using a three-axis vector magnet. Transport measurements were performed by using standard ac lock-in techniques in a dilution refrigerator, with a base temperature of 20 mK.

Differential conductance measured for device 1 is shown in Fig. 1C as a function of source-drain voltage, V_{sd} , between the normal and superconducting leads, and the voltage, V_{g1} , on gate $g1$. The height (in V_{sd}) of the Coulomb-blockade diamond yields an end-dot charging energy $E_c \sim 6$ meV. Because E_c is larger than the superconductor gap, single-electron cotunneling dominates transport in Coulomb-blockade valleys. In this regime, the dot acts effectively as a single barrier and can be used as a tunneling spectrometer for the wire (Fig. 1E). On the other

hand, when the dot is tuned onto a Coulomb peak (Fig. 1F), hybridization occurs between the dot and wire states (36). We first discuss cotunneling spectra away from resonance then investigate dot-wire interaction when the dot is on resonance with ABSs and MBSs in the wire.

Weak dot-wire coupling

A hard proximity-induced superconducting gap, marked by vanishing conductance below coherence peaks, can be seen in cotunneling transport through Coulomb blockade valleys of the end dot (Fig. 1D). The width of the gap in bias voltage is given by $2\Delta^*/e$, where Δ^* is the effective superconducting gap, defined phenomenologically by the bias voltage at which the

quasiparticle continuum appears. The value of Δ^* for device 1 is found to be $220 \mu\text{eV}$ (for devices 2, 3, and 4, $\Delta^* \sim 250$ to $270 \mu\text{eV}$), which is somewhat larger than measured previously in either epitaxial (33) or evaporated hybrid devices (27, 37). The measured gap is consistent with values for evaporated ultrathin Al films in the literature (38).

Tunneling conductance (dI/dV_{sd}) for device 1, as a function of V_{g1} and V_{sd} , spanning three Coulomb blockade valleys is shown in Fig. 2 for two values of back-gate voltage V_{bg} , which is applied uniformly to the device by using a conductive Si substrate separated by a 200-nm oxide layer. To compensate the effect of V_{bg} on the conductance of the end dot, the voltage V_{g1} , on the gate near the end dot is simultaneously swept by a small amount during the back-gate sweep. Other gates are grounded. At less negative back-gate voltage ($V_{bg} = -2.5$ V), several subgap conductance peaks are seen at $B = 1$ T, including one at zero bias. We attribute these peaks, which run through consecutive Coulomb valleys, to ABSs in the finite-length wire. The magnetic field dependence of the spectrum is shown in Fig. 2, C and D: subgap states lie close to the superconducting gap at zero field and move to lower energies as B increases. Some of the lower-energy subgap states merge at zero energy, forming a narrow zero-bias peak spanning the range from 1 to 2 T. At more negative back-gate voltage, $V_{bg} = -7$ V, dot-independent subgap structure is absent (Fig. 2, E to H); only a hard superconducting gap is seen throughout the field range of 0 to 2 T. The back-gate dependence on the number of ABSs in the gap demonstrates that the chemical potential of the wire can be controlled with the superconductor shell present.

The zero-field effective gap Δ^* in the regime with high ABS density is $\sim 200 \mu\text{eV}$, which is distinctly smaller than the $220\text{-}\mu\text{eV}$ gap seen in the no-ABS regime. This is because the phenomenological Δ^* in the high-ABS density regime is mainly determined by the energy of the cluster of ABSs, yielding what is usually referred to as the induced gap Δ_{ind} . When there are no states in the wire, Δ^* is set by the gap of the Al shell, denoted Δ .

Between the regimes of high ABS density and zero ABS density, one can find, by adjusting back and local gates, a low-density ABS regime in which only one or a few subgap modes are present. In this intermediate density regime, ABSs can be readily probed with tunneling spectroscopy, without softening the gap with numerous quasicontinuous subgap states. To prevent end-dot states from mixing with ABSs in the wire, two gate voltages, one at the junction and one along the wire, were swept together so as to compensate for capacitive cross-coupling (Fig. 3A). In this way, either the end-dot chemical potential μ_{dot} or the wire chemical potential μ_{wire} could be swept, with the other held fixed. A two-dimensional plot of zero-bias conductance as a function of V_{g1} and $V_{g2,g3}$ (fixing $V_{g2} = V_{g3}$) in Fig. 3B shows isopotential lines for the end dot

as a diagonal Coulomb blockade peak-ridge (red arrows). The slope of this ridge determines how to compensate the wire gates ($V_{g2,g3}$) with the junction gate (V_{g1}). Data can then be taken in the cotunneling regime for an effectively constant μ_{dot} . Tunneling spectra measured along the red line in Fig. 3B at various fields are shown in Fig. 3, C to F. A pair of ABSs that moves with μ_{wire} can be seen at $B = 0$ (Fig. 3C). The spectrum is symmetric around zero V_{sd} , reflecting particle-hole symmetry. The minimum energy of the ABS is $\zeta = 130 \mu\text{eV}$, which is smaller than the effective gap $\Delta^* = 220 \mu\text{eV}$. The pair of ABSs splits into two pairs when the applied magnetic field lifts the spin degeneracy, visible above $B = 0.4 \text{ T}$ (Fig. 3D). The low field splitting corresponds to an effective g -factor, $g^* \sim 4$ (the g^* -factor estimated from the ABS-energy/magnetic field slope may differ considerably from the intrinsic g^* -factor). At higher magnetic fields, the inward ABSs cross at zero and reopen, forming a characteristic oscillatory pattern (Fig. 3, E and F). The gap reopening at more positive $V_{g2,g3}$ is relatively slow, leading to a single zero-bias peak in the range of $V_{g2,g3} \sim 5.8$ to 7 V (Fig. 3F).

The magnetic field dependence of the ABS spectrum near the ABS energy minimum is shown in Fig. 3G. The evolution of the ABSs can be clearly followed: They split at low field, the inner ABSs merge around $B = 1 \text{ T}$, they split again at higher fields, and the resplit ABSs

merge with the higher-energy ABSs above $B = 1.7 \text{ T}$. Here, the emergence of a zero-bias peak and its splitting is qualitatively similar to the observations reported in (27, 30). However, the B -dependent ABS spectrum at more positive gate voltage (Fig. 3H) shows a merging-splitting-merging behavior, giving rise to an eye-shaped loop between 1 and 2 T. At even more positive gate voltage (Fig. 3I), the spectrum displays an unsplit zero-bias peak from 1.1 to 2 T. The first excited ABSs in Fig. 3, G to I, are still visible at a high magnetic field—for instance, as marked at $B = 1.2 \text{ T}$ in Fig. 3, H and I. Qualitatively, the lowest-energy ABSs in Fig. 3, H and I, tend to split after crossing but are pushed back by the first excited ABSs, resulting in either a narrow splitting or an unsplit zero-bias peak. The measurements in Fig. 3, C to I, were taken in an even Coulomb valley of the end dot, but the qualitative behavior does not depend on end-dot parity. Similar results measured in an odd valley of the end dot are provided in (35).

The different field dependences of the subgap states—either crossing zero or sticking at zero—can be understood as reflecting a transition from ABS to MBS (14, 17). For the ABSs in the regime of $V_{g2,g3} < 5.8 \text{ V}$, their crossing in Zeeman field is a signature of parity switching, similar to ABSs in a quantum dot, such as investigated in (30). In contrast, behavior in the range of $V_{g2,g3} \sim 5.8$ to 7 V indicates that the system is in the topologically nontrivial regime, with MBS levels that

stick to zero as the magnetic field increases. In a finite-size wire, SOI induces anticrossings between discrete ABSs, thus pushing levels to zero, preventing further splitting. We ascribe the differences in the qualitative behavior in Fig. 3, G to I, to state-dependent SOI-induced anticrossings, which depend on gate voltage. The excited ABS in Fig. 3G and the ones in Fig. 3, H and I, are presumably not the same state, but belong to different subgap modes [investigated in detail in (35)].

For a long wire, the topological phase transition is marked by a complete closing and reopening of a gap to the continuum, with a single discrete state remaining at zero energy after the reopening. For a finite wire, the continuum is replaced by a set of discrete ABSs, and at the transition where a single state becomes pinned near zero energy, there remains a finite gap δ to the first discrete excited state. At this transition point (where the gap of the corresponding infinite system would close and its spectrum would be linear), $E_k = R\alpha|k|$, where R is a renormalization factor due to the strong coupling between the semiconducting wire and its superconducting shell (35), α is the spin-orbit coupling strength, and k is the electron wave vector. From this relation, we can connect δ to the ratio L/ξ as $L/\xi \approx R\pi\Delta^*/\delta$, where L is the separation between Majoranas (the wire length in the clean limit), ξ is the effective superconducting coherence length near the topological

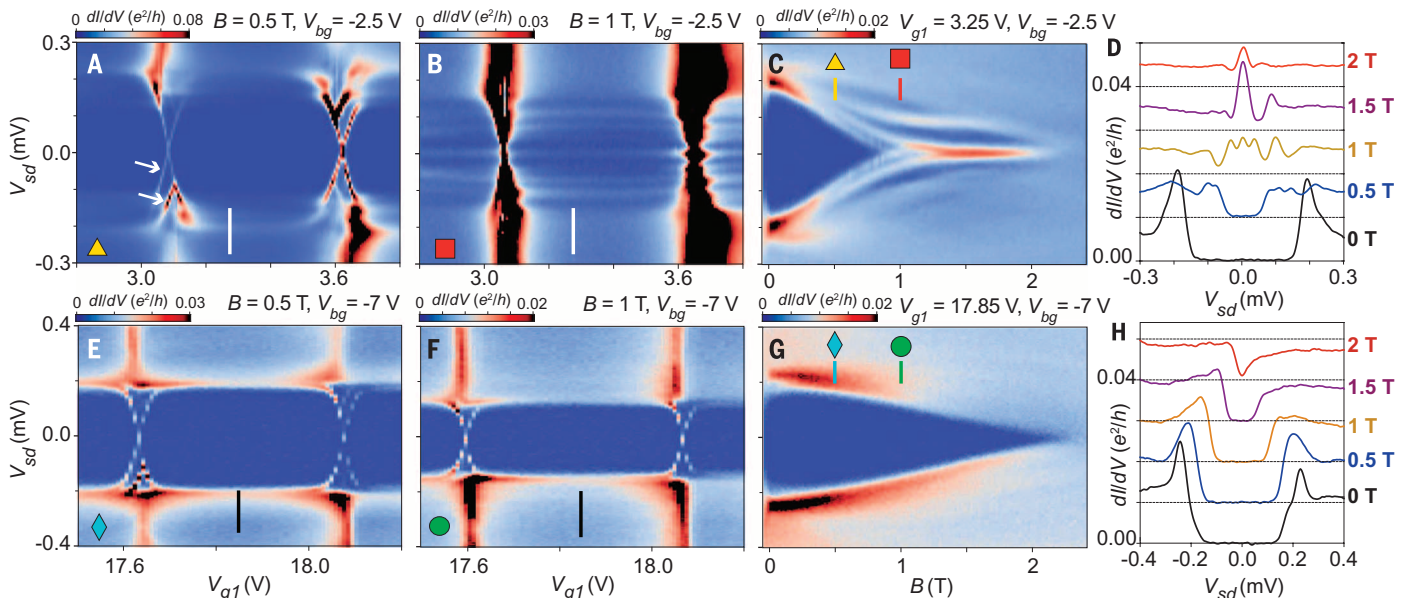


Fig. 2. Tunneling spectra for large and zero ABS density. (A) Differential conductance measured for device 1 as a function of V_{sd} and V_{g1} , measured at $B = 0.5 \text{ T}$ and $V_{bg} = -2.5 \text{ V}$, $V_{g2,g3} = -10 \text{ V}$. The white arrows indicate Zeeman split dot levels. (B) The same as (A), but at $B = 1 \text{ T}$. ABSs can be clearly identified below the superconducting gap. (C) Differential conductance as a function of V_{sd} and B (B - V_{sd} sweep), measured at the gate voltage indicated by the white lines in (A) and (B). The triangle and square indicate at which fields (A) and (B) are measured, respectively. Blurring of data in narrow $B < 0$ range is due to heating caused by sweeping field away from zero. (D) Line-cut

taken from (C) at various B values. Lines are offset by $0.01 e^2/h$ each for clarity. The conductance peaks below the superconducting gap indicate that the wire is in a subgap-state-rich regime. A well-defined zero-bias peak can be seen at high field. (E to H) Similar to (A) to (D), but measured at $V_{bg} = -7 \text{ V}$ and $V_{g2,g3} = -10 \text{ V}$, and with (G) measured at the gate voltage indicated by the black lines in (E) and (F). The diamond and circle indicate at which fields (E) and (F) are measured, respectively. Here, a hard superconducting gap is clearly seen, with a critical magnetic field B_c up to $\sim 2.2 \text{ T}$. No subgap structure is observed across the full range of field, 0 to 2 T.

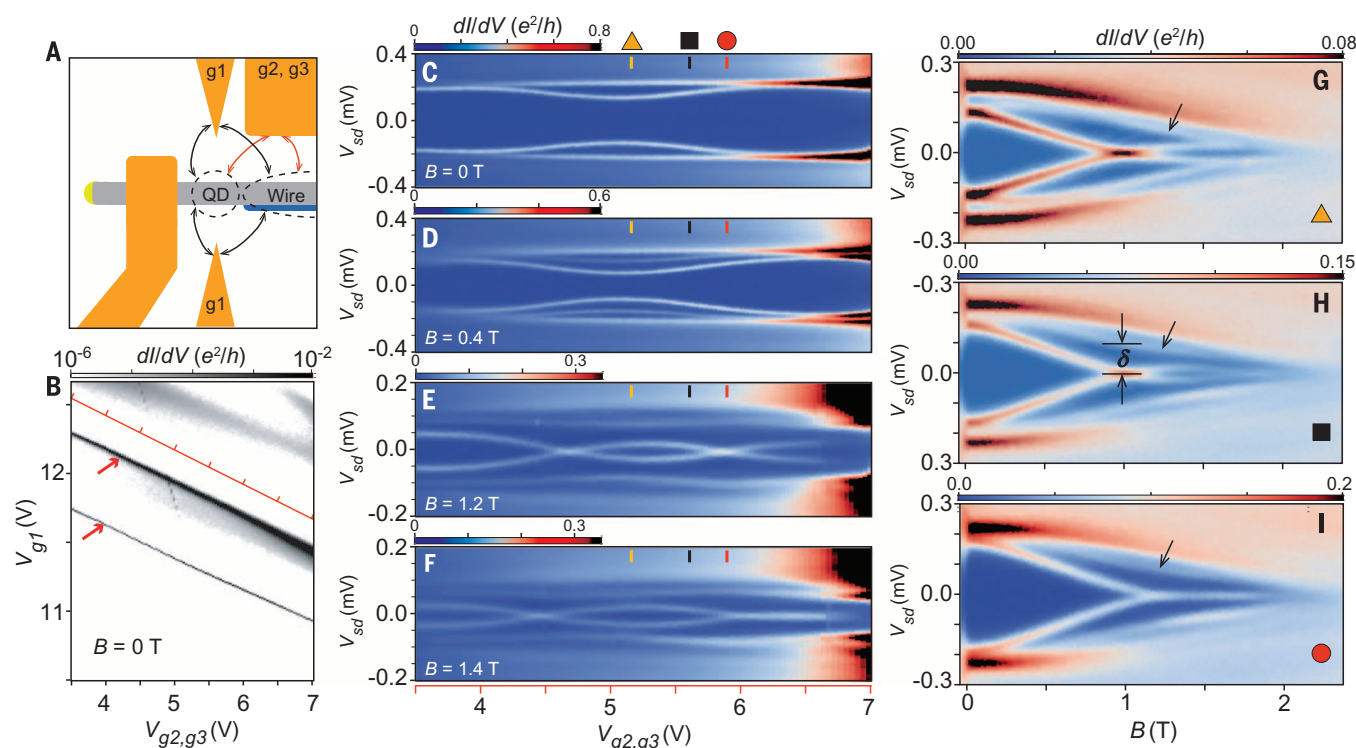


Fig. 3. Tunneling spectra for intermediate density in few-ABS regime.

(A) A schematic of device 1 showing the gating configuration for a combined gate voltage sweep. g_1 and g_2, g_3 are capacitively coupled to both the dot and the nanowire. (B) Conductance measured at $V_{sd} = 0$ mV, $V_{bg} = -7$ V, and $B = 0$, as a function of V_{g1} and $V_{g2,g3}$ (the gate map). g_2 and g_3 are connected to the same voltage source. The high-conductance lines indicated by red arrows are the resonant levels in the end dot. The dot can be used as a cotunneling spectrometer if the gate sweeping is kept inside the Coulomb blockade valley and parallel to the resonant level. (C to F) Tunneling spectra at various

magnetic fields as a function of the combined gate voltage, measured along the red line in (B). The energy of the ABSs is strongly dependent on gate voltages. (G to I) B - V_{sd} sweeps at different gate voltages, corresponding to the triangle, square, and circle in (C) to (F), respectively. Depending on gate voltages, the ABSs in the wire show different magnetic field evolution, from a splitting behavior (G) to non-splitting behavior (I). Arrows in (G) to (I) indicate the first excited ABSs, and δ in (H) is defined as the residual gap—the energy of the first excited state around topological phase transition, caused by the finite-size effect.

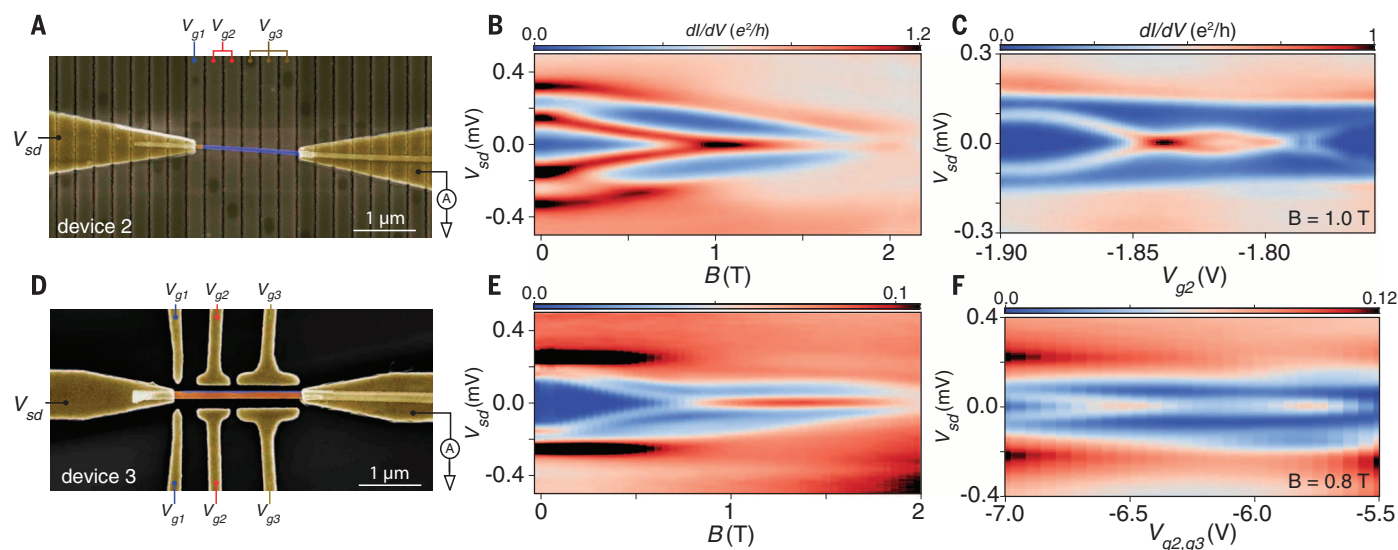


Fig. 4. Stable zero-energy states measured on other devices. (A) SEM images of device 2, in which local bottom gates are used. The hybrid wire section is $1.5 \mu\text{m}$ long. (D) SEM image of device 3, with the hybrid wire section length around $2 \mu\text{m}$ long. (B and E) Subgap states evolution in magnetic field, measured on device 2 and device 3, respectively. In both plots, stable zero-energy states arising from a pair of ABSs can be seen.

(B) is measured at $V_{g1} = -600$ mV, $V_{g2} = -1840$ mV, and $V_{g3} = 5$ V, and (E) is measured at $V_{g1} = 3720$ mV, $V_{g2} = V_{g3} = -5850$ mV, and $V_{bg} = -8$ V. (C and F) Gate voltage-dependence measurements of subgap states for device 2 and device 3, respectively. Both measurements are taken by following the isopotential lines of the hybrid wires in one of their end-dot Coulomb blockade valleys.

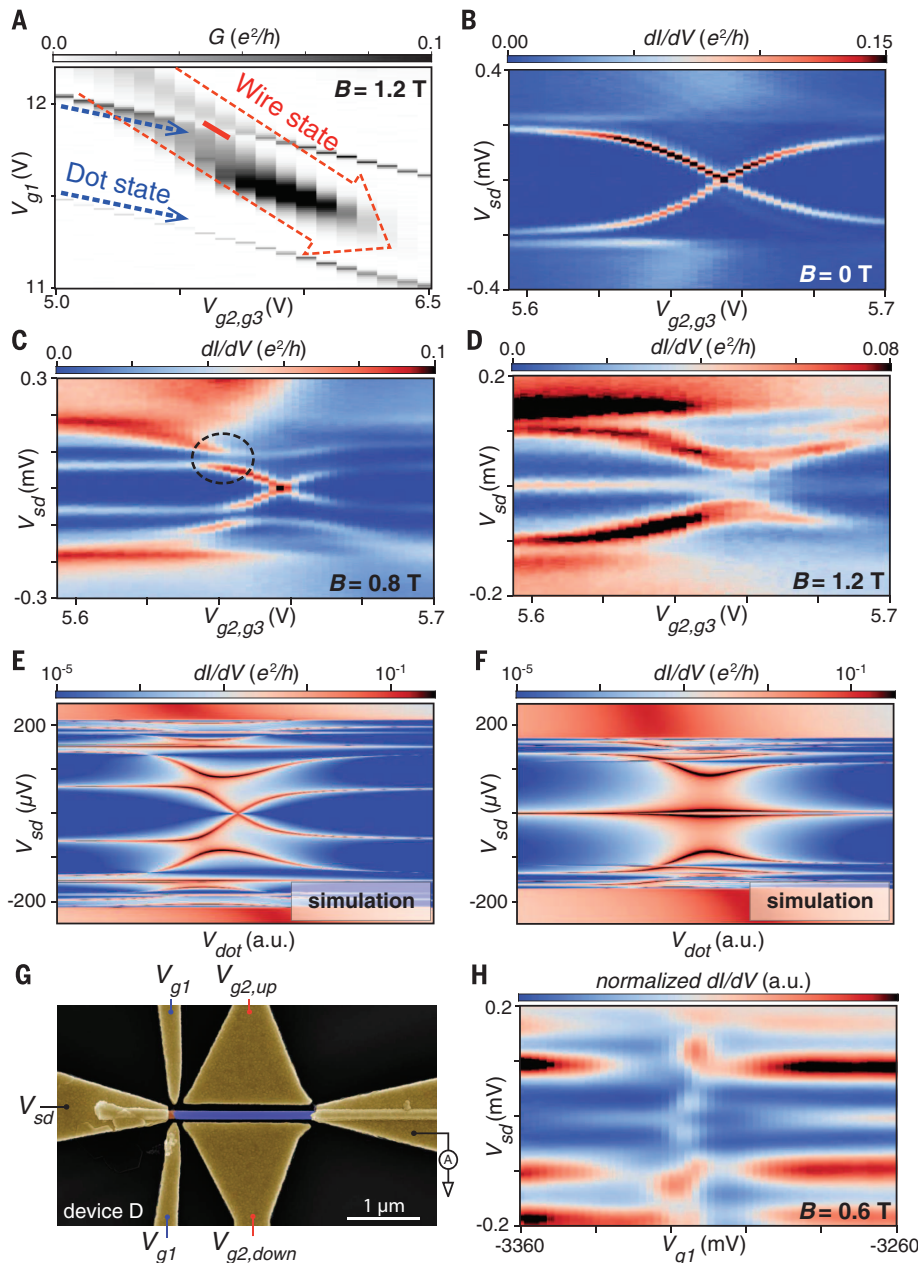


Fig. 5. Resonant coupling of wire subgap states and dot states. (A) A gate map similar to Fig. 3B, but taken at $B = 1.2$ T. The blue dashed arrows denote the dot isopotential sweeping direction, and the red dashed arrow denotes the wire isopotential sweeping direction. (B to D) Differential conductance measured across one of the resonant dot levels, along the solid red line in (A), at $B = 0, 0.8$, and 1.2 T. The dashed circle in (C) indicates an anticrossing between the dot state and a wire state. In (D), there is a pronounced zero-bias peak-splitting at the dot resonance. (E and F) Simulated differential conductance through a dot-hybrid wire system, as a function of bias voltage and dot chemical potential μ_{dot} , for different Zeeman splittings in the wire. The zero-bias peak splitting at the dot resonance also appears in (F). (G) SEM image of device 4. (H) Normalized conductance in the MBS-dot hybridization region of device 4. Again, the zero-energy MBS state is split when it crosses the end-dot resonant level.

phase transition, and Δ' is the effective gap near the phase transition point [the derivation and more details are available in (35, 39)]. The ratio L/ξ is the dimensionless length of the topological wire segment. We estimate from Fig.

3H $\delta \sim 100$ μeV , $\Delta' \sim 180$ μeV , and $R \sim 0.4$, yielding $L/\xi \sim 2.3$. We then take values at the field where the ABS reaches zero energy. We can independently estimate L/ξ from the relation $\delta E \approx \delta E_0 e^{-L/\xi}$ (14, 17), where δE is MBS

oscillation amplitude and δE_0 is a prefactor. If we take the value $\delta E_0 \sim 150$ μeV based on Coulomb peak motion in (40), we obtain a value $L/\xi \sim 1.3$ by using the subgap state splitting energy $\delta E \sim 40$ μeV at $B \sim 1.3$ T from Fig. 3H. We speculate that the discrepancy in estimates of L/ξ may be attributed to a smaller value of δE_0 in (40) as compared with the δE_0 in this device, perhaps arising from differences in gate-tuned electron blockade density compared with the Coulomb blockade devices in (40).

Subgap-state evolution in applied magnetic field and gate voltage for devices 2 and 3 are shown in Fig. 4. For device 2, which has a device length of ~ 1.5 μm , subgap state reaches zero energy at ~ 0.9 T and persists to 2 T. Using the finite-size gap near the phase transition point in Fig. 4B, we can extract $L/\xi \sim 1.7$ ($\delta \sim \Delta' \sim 170$ μeV , $R \sim 0.56$). For device 3, with a ~ 2 μm hybrid nanowire, rigid zero energy states are shown in both magnetic field and gate voltage-dependence measurements. Here, a value of $L/\xi \sim 3.4$ ($\Delta' \sim 230$ μeV , $\delta \sim 100$ μeV , and $R \sim 0.47$) can be estimated from Fig. 4E.

The obtained values for L/ξ evidently do not reflect the lithographic device length. For instance, taking $\xi \sim 260$ nm from (40) (where ξ is fit from the wire-length dependence by using similar nanowires), yields lengths of 0.6, 0.45, and 0.9 μm for devices 1, 2, and 3, respectively, in each case shorter than the lithographic length. This discrepancy is presumably the result of disorder or material defects that create a topological region shorter than the full wire.

Resonant dot-MBS coupling

We next examined the interaction of wire states with bound states in the end dot. For all data above, the wire states were probed via cotunneling through the end dot (Fig. 1E), actively keeping the end dot in the middle of a Coulomb valley. By separately controlling μ_{dot} and μ_{wire} , we can also tune the local gates so that a wire state is at resonance with a Coulomb peak of the dot (Fig. 1F). As seen in the gate map for device 1 shown in Fig. 5A, besides the dot resonant level, there is a MBS-induced blurred trace (Fig. 5A, red dashed arrow). Tunneling spectra at various magnetic fields where the MBS and end-dot state align are shown in Fig. 5, B to D [traces with a larger range are provided in (35)]. The gate sweep is now along a wire isopotential (Fig. 5A, red solid line) as opposed to an end-dot isopotential as in Fig. 3. The dot state crosses zero energy around $V_{g2,g3} = 5.66$ V, at which the dot switches its fermion parity. At $B = 0$ (Fig. 5B) and at $B = 0.8$ T (Fig. 5C), we clearly see this crossing in the spectrum. There is a pronounced anticrossing between the dot state and the wire state in Fig. 5C (indicated by the dashed circle). Figure 5D looks qualitatively different: A zero-bias peak is visible when the dot is off resonance (which is extended from the MBS in Fig. 3H), and this peak splits when the dot level comes close to zero. In this case, no zero-crossing is observed.

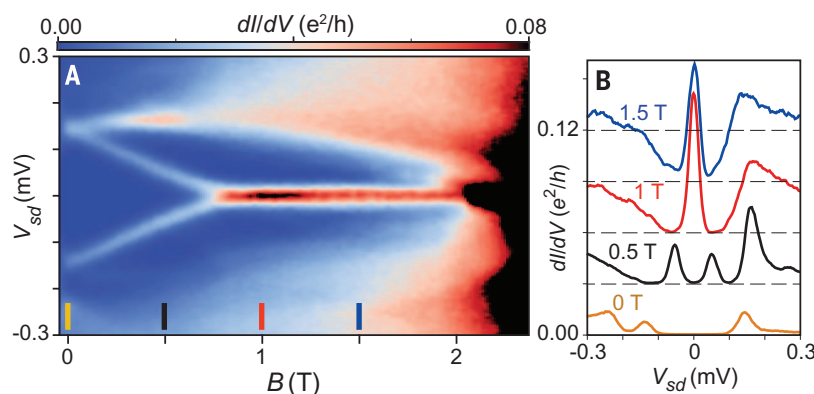


Fig. 6. Tunneling spectrum for resonant dot-wire coupling. (A) B - V_{sd} sweep at $V_{bg} = -8.5$ V, $V_{g1} = 22$ V, and $V_{g2} = V_{g3} = -10$ V. (B) Differential conductance line-cut plots taken from (A) at various B values. At this gate configuration, a pronounced zero-bias conductance peak emerges around $B = 0.75$ T and persists above $B = 2$ T, without splitting. The intensity of the zero-bias peak is higher than other finite-energy ABSs and even higher than the Al superconducting coherence peaks. The background conductance is almost zero even at $B = 1$ T, indicating that the induced gap is still a hard gap after the phase transition.

The dot-wire interaction observed in Fig. 5D can be understood in terms of leakage of the MBS into the dot when the dot is on resonance ($4I$). The energy splitting of a pair of MBSs is given by $\delta E \propto |\sin(k_F L) e^{-L/\xi}|$ (where k_F is the effective Fermi wave vector). In Fig. 5D, this splitting is initially small, when the dot is off resonance and coupling of the MBSs to the dot states is suppressed by Coulomb blockade. For a finite-size wire, this implies that $\sin(k_F L) \sim 0$ at that particular tuning. As the dot level comes closer to the resonant point, the nearby MBS partially leaks into the dot, which changes the details of the MBSs wave function [the numerical study on the wave-function distribution is provided in (35)]. This can change the effective $k_F L$ in δE , which causes the zero-bias peak to split at resonance. Numerical simulations of the conductance spectrum of the coupled dot-MBS (Fig. 5, E and F) show good qualitative agreement with the experimental data, both in the trivial superconducting regime (Fig. 5, C and E) and in the topological superconducting phase (Fig. 5, D and F). Similar zero-bias peak splitting in another coupled dot-MBS device (device 4) is shown in Fig. 5H. To enhance image visibility, conductance values in Fig. 5H are normalized by the conductance at $V_{sd} = 0.2$ mV at the corresponding gate voltage.

Last, we examined the magnetic field evolution of the subgap states in the strong dot-wire coupling regime, in which dot and wire states cannot be separated. Shown in Fig. 6 is the evolution with field of the spectral features of the dot-wire system measured for device 1, with two ABSs merging at $B = 0.75$ T into a stable zero-bias peak that remains up to $B = 2$ T. The effective g^* -factor that can be deduced from the inward ABS branches is ~ 6 . The conductance at the base of the zero-bias peak is almost zero even at $B = 1$ T, indicating a hard superconducting gap also after the topological phase

transition. Related measurements are shown in (35).

The long field range and intensity of the zero-bias peak in Fig. 6 can be understood as arising from the hybridization of the MBS with the end-dot state. In the strong coupling regime, MBS can partially reside at the end dot, making the effective length of the wire longer than in Fig. 3I. The MBS wave function has larger amplitude at the wire end, where the dot couples, than either finite-energy ABSs or states in the Al shell. This leads to a relatively higher conductance peak at zero energy and makes the excited states and the Al shell superconducting coherence peaks almost invisible (13). The long field range of the zero-bias peak in Fig. 6 (also Fig. 3I) may also be enhanced by electrostatic effects that depend on magnetic field (14, 19).

Our measurements have revealed how the ABSs in a hybrid superconductor-semiconductor nanowire evolve into MBSs as a function of field and gate voltage.

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SUPPLEMENTARY MATERIALS

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Supplementary Text
Figs. S1 to S11
References (42–46)

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COMETARY SCIENCE

Seasonal exposure of carbon dioxide ice on the nucleus of comet 67P/Churyumov-Gerasimenko

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Carbon dioxide (CO₂) is one of the most abundant species in cometary nuclei, but because of its high volatility, CO₂ ice is generally only found beneath the surface. We report the infrared spectroscopic identification of a CO₂ ice-rich surface area located in the Anhur region of comet 67P/Churyumov-Gerasimenko. Spectral modeling shows that about 0.1% of the 80- by 60-meter area is CO₂ ice. This exposed ice was observed a short time after the comet exited local winter; following the increased illumination, the CO₂ ice completely disappeared over about 3 weeks. We estimate the mass of the sublimated CO₂ ice and the depth of the eroded surface layer. We interpret the presence of CO₂ ice as the result of the extreme seasonal changes induced by the rotation and orbit of the comet.

On comets, sublimation caused by solar illumination is the major mechanism that differentiates volatile species over their long dynamical lifetimes. The penetration of heat into the comet's nucleus causes chemical stratification, with more volatile molecules, such as CO and CO₂, receding into the interior ices, whereas less volatile molecules, such as water, remain close to the surface and enrich the near-surface ices (1). The seasonal variability associated with the relatively large orbital eccentricities of comets is further complicated by the irregular shape of their nuclei and the inclination of their rotational axes, which amplify the seasonal effects. Thus far, thermal evolution models have not provided evidence of recondensation or fast sublimation of volatile species following an extreme seasonal cycle. Evidence from the comet 67P/Churyumov-Gerasimenko (67P/CG) confirmed

the general volatiles stratification scheme during the preperihelion period, when the spatial distribution of water vapor and CO₂ in the gaseous coma was interpreted as the result of CO₂ ice sublimating from inner layers, with water ice sublimating closer to the surface (2–6). A diurnal cycle of water ice has been observed in which an ephemeral frost layer forms in response to sudden shadowing conditions and day-night variation (7).

In early 2015, the southern hemisphere of 67P/CG was emerging from shadows and leaving its cold season, during which subsurface temperatures were as low as 25 to 50 K (8). In particular, an area located in the Anhur region [at longitude 66.06° and latitude –54.56° in Cheops coordinate frame (9)] had experienced a 4-year-long winter season before being illuminated by the Sun in mid-January 2015.

Using the VIRTIS-M (Visible, Infrared and Thermal Imaging Spectrometer, Mapping Chan-

nel) onboard the Rosetta spacecraft (10), we detected a CO₂ ice-rich area in the Anhur region on 21–22 March 2015, when the comet was 2.05 astronomical units (AU) from the Sun. This region is 80 by 60 m (resolved at 20 m per pixel in VIRTIS-M images) and is shown in the general context of the nucleus digital shape model in Fig. 1. The deposit is located in a smooth area of the Anhur region, on the large lobe of the nucleus (11). The same spectral data set, for which we give additional details in (12), has been used to derive quantitative information on CO₂ abundance. The irradiance/solar flux (I/F) spectrum of the pixel with the most intense absorption features is shown in Fig. 2. The I/F spectrum is characterized by a steep red spectral slope up to 1.5 μm; an absorption triplet at 1.97, 2.01, and 2.07 μm; and two further absorptions at 2.7 and 2.78 μm. These features correspond to known absorption features of CO₂ and thus indicate the presence of CO₂ ice on the surface of 67P/CG. The CO₂ ice fundamental band at 4.26 μm is not visible in the I/F spectra because of the thermal emission from the nucleus. Detailed descriptions of the spectral variability across the CO₂ ice-rich area, the removal of the thermal emission signal, and the spectral modeling are given in (12).

The spectra of the CO₂ ice-rich area have been fitted with a radiative transfer model (13, 14), allowing us to derive quantitative information on end-member abundances, mixing modalities, and grain sizes. The I/F spectrum is simulated by using areal and intimate mixtures of two components: the dark terrain (DT) unit, corresponding to the measured average organic-rich spectrum of the comet's surface (15) after the application of photometric correction (16), and CO₂ ice derived from optical constants (17, 18). The composition of the DT unit, characterized by low albedo, a red slope, and a broad absorption feature in the 3.2-μm range, is still uncertain (19). To check the quality of the spectral retrieval, we performed an additional fit by replacing CO₂ ice with crystalline water ice (20–22). The VIRTIS spectral mixing and data processing methods are described at length in previous works (23, 24). The modeling of each individual pixel in which CO₂ ice was detected is discussed in (12).

The results of the spectral modeling are shown in the left panel of Fig. 2. The best-fitting model including a water ice component requires 99.7% DT with a slope (necessary to correct for photometric response) of –3.8% μm^{–1}, plus 32-μm water ice grains in areal mixing for the remaining 0.3%. The quality of this fit is very poor, however (χ² = 3.85), in particular in the 2.0-μm and 2.7- to 2.8-μm ranges, where water ice is

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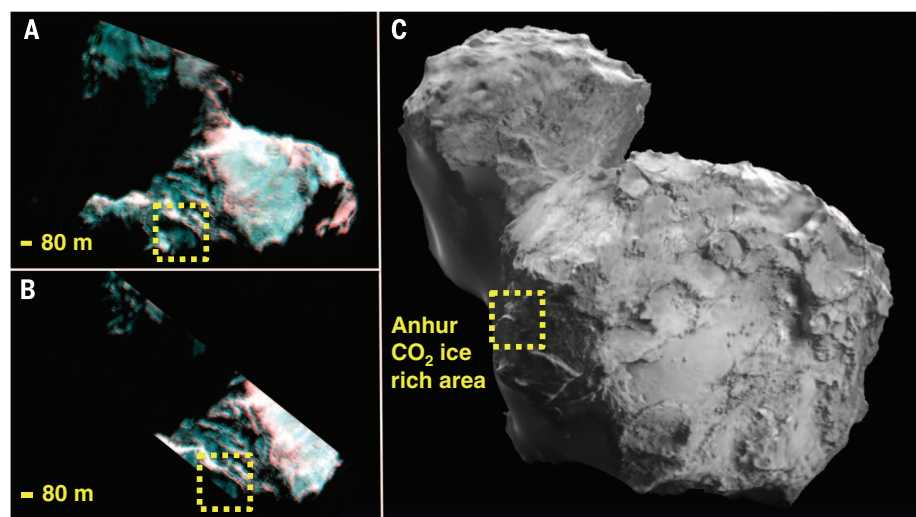


Fig. 1. VIRTIS-M infrared images of the Anhur CO₂ ice-rich area, 21–22 March 2015. The center of the yellow dashed box is the location of the CO₂-rich patch in VIRTIS-M images (A) I1_00385598211 and (B) I1_00385688463, as well as (C) on the nucleus shape model (from <http://sci.esa.int/comet-viewer/?model=esa>). VIRTIS-M color images are a RGB combination of infrared bands (blue at 1.2 μm , green at 2.0 μm , and red at 4.0 μm). A description of the VIRTIS-M acquisitions is given in (12).

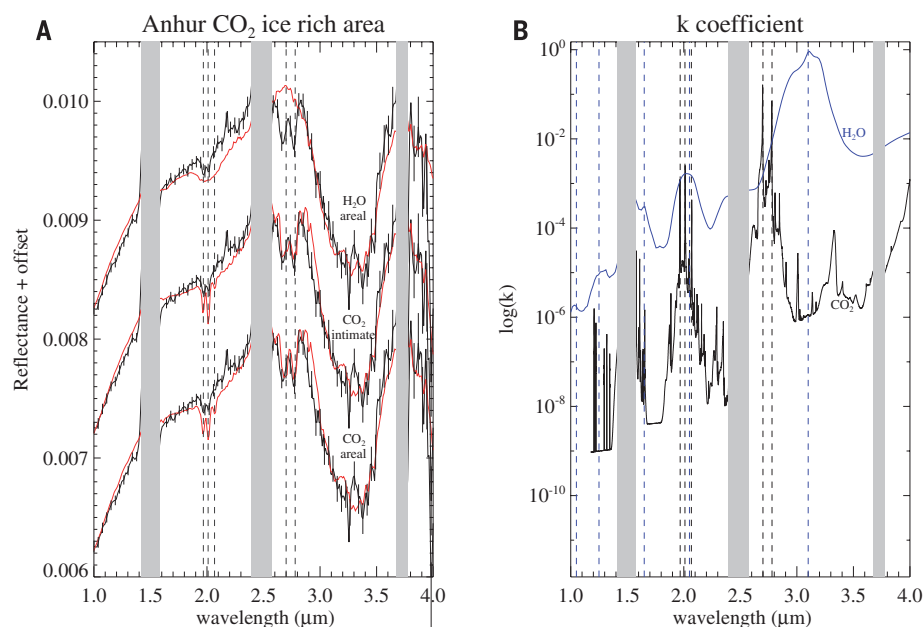


Fig. 2. Spectral modeling of the CO₂ surface ice from the 21 March 2015 observation. (A) VIRTIS-M reflectance (black curve, from observation I1_00385598211; pixel $s = 126$, $l = 109$) and spectral simulations carried out with dark terrain (DT) and H₂O ice in areal mixing (top, plus 0.002 offset), DT and CO₂ ice in intimate mixing (center, plus 0.001 offset), and DT and CO₂ ice in areal mixing (bottom) for the Anhur CO₂ ice-rich area. Spectral intervals shaded in gray correspond to data that are missing as a result of instrumental order sorting filters. Error bars, shown every five bands to improve readability, give the instrument noise on the pixel. (B) Imaginary part k of refractive indices for H₂O ice (blue curve) and CO₂ ice (black curve). Optical constants for CO₂ are from (17,18) and for H₂O are from (20–22). Vertical lines mark the k local maxima corresponding to the strongest absorption bands at 1.05, 1.25, 1.65, 2.05, and 3.10 μm for H₂O ice (in blue) and at 1.97, 2.01, 2.07, 2.70, and 2.78 μm for CO₂ ice [in black; also shown in (A)].

not able to reproduce the observed absorption features, indicating its absence in this area. Better fits are achieved by replacing water with CO₂ ice (Fig. 2). We show two possible models. The first corresponds to an intimate mixing (12) of 99.2% DT with a $-5.12\% \mu\text{m}^{-1}$ slope, plus 0.8%

800- μm CO₂ ice grains. The second case corresponds to an areal mixing of 99.9% DT with a slope of $-4.76\% \mu\text{m}^{-1}$, plus 0.1% 50- μm CO₂ ice grains. With a residual of $\chi^2 = 2.05$, the areal solution appears marginally better than the intimate, for which $\chi^2 = 2.45$. The addition of water

ice to the components of the mixture does not improve the fit quality. In both models, less than 1% CO₂ ice is sufficient to account for the observed spectral absorption features.

The CO₂ ice features were detected on 2 consecutive days (21 and 22 March 2015). This means that the CO₂ ice area was stable against day-night variations in temperature. However, after the March detection, VIRTIS-M did not observe the Anhur region again until 12–13 April 2015, when the heliocentric distance was reduced to 1.87 AU and the pixel scale was reduced from 20 to 39 m per pixel. At this time, the spectra acquired did not show any of the previously observed CO₂ absorption features (Fig. 3), indicating that the CO₂ ice patch had diminished to a level below the detection limit of the VIRTIS-M instrument (band depth less than 1% relative to the local continuum, corresponding to $<0.1\%$ CO₂ ice abundance), if not disappeared altogether. The reduction of the spatial resolution by a factor of about 2 and the increase of the solar phase angle from 54° to 79° between the two sets of observations must be considered in interpreting the disappearance of the CO₂ ice spectral features: Apart from the change in spatial resolution, the last images are more affected by long shadows caused by local topography, making the detection of the CO₂ ice more difficult. Conversely, assuming a uniform distribution of CO₂ ice in areal mixing with the DT within the initial 80- by 60-m area, one should expect to also detect it on the less resolved images because the instrumental resolution is still within the size of the area of interest.

The maximum surface temperature, derived by modeling the thermal emission at 4.5 to 5.0 μm with a Bayesian method (25), increased in this area from 218.8 K on 21 March 2015 to 225.9 K on 13 April 2015. However, given that the surface is not isothermal at the subpixel scale because of local roughness and shadows, these should be considered as upper limits, representative only of the warmest fractions of the pixel, corresponding to the more illuminated subpixel areas. The measured temperatures are well above the sublimation temperature of CO₂ ice, which is about 80 K (26); therefore, the CO₂ ice-rich region cannot be in thermal equilibrium with the DT, and continuous sublimation must occur. For this reason, intimate mixing between CO₂ ice and DT is much less favorable than areal mixing. As a result of the sublimation of the volatile species on the surface, an increase in the local roughness of the more illuminated areas is very likely to occur.

We investigated the illumination history of the Anhur CO₂ ice-rich area by tracing the variation of the incident solar flux during the time for which VIRTIS-M observations are available. Throughout the period from January 2011 to January 2015, the Anhur region was in permanent shadow, whereas from 14 January 2015 forward, the combined effect of decreasing heliocentric distance and longer insolation caused a net increase of solar flux to the area (Fig. 4). After scaling the instantaneous solar flux with the cosine of the incidence angle calculated using the nucleus

digital shape model (27, 28), we found that at the time of the CO₂ ice detection (21–22 March 2015), the maximum solar flux at local noon was about 80 W/m² (Fig. 4). At the time of the second series of VIRTIS-M observations, the maximum solar flux at local noon had increased to about 135 W/m².

The integrated solar flux accumulated by an area of 0.4 m², corresponding to the CO₂ areal occupancy within the 400-m² VIRTIS-M pixel, is equal to 2.026×10^7 J over the 45 rotations of the comet occurring between the available VIRTIS-M observations. Assuming a latent heat of sublimation of 552 kJ/kg for CO₂ ice (26) and neglecting the thermal conduction in view of the very low thermal inertia reported for 67P/CG (8, 29), we derive a value for the total amount of sublimated ice of 35 kg, equivalent to the erosion of a 5.6-cm-thick layer [further details are given in (12)]. This value is an upper limit, assuming a complete sublimation of the ice during 45 rotations. A similar estimate, performed from the beginning of the illumination conditions in early January 2015 until the time of the CO₂ ice detection, gives an additional sublimated ice mass of 22 kg. Hence, the area within a VIRTIS-M pixel experienced a maximum sublimation of 57 kg of CO₂ ice, corresponding to the erosion of a 9-cm layer, in about 3 months. The temporal trend of the cumulative CO₂ ice sublimation mass is shown in Fig. 4.

This amount of sublimated CO₂ ice is too small to contribute meaningfully to the gaseous coma emissions. Above the southern hemisphere, VIRTIS has detected a CO₂/H₂O ratio of about 4% (4), but no evidence has been found in VIRTIS-M spectra of increased H₂O or CO₂ gaseous activity in the surroundings of the Anhur region. In fact, even assuming a sublimation rate of 1 kg of CO₂ ice per day from a 20- by 20-m area, the resulting column density of 10^{17} m⁻² in the ambient coma is about two orders of magnitude lower than the average value of 10^{19} m⁻² measured by VIRTIS-M (4).

The observation of the CO₂ ice-rich spot was unexpected at these heliocentric distances, given the high volatility of CO₂; water ice, a less volatile species, was not observed in the same region at the same time, as confirmed by the spectral fit shown in fig. S5 (12). 67P/CG falls in the ensemble of the CO₂-rich comets, as demonstrated by the high activity level of CO₂ above the southern hemisphere, where the CO₂/H₂O ratio is considerably larger than in the northern hemisphere (4, 30).

The OSIRIS (Optical, Spectroscopic, and Infrared Remote Imaging System) instrument did observe water ice in this same region (31) in April–May 2015, about 6 weeks after the initial detection of the CO₂ ice by VIRTIS-M. At this time (by 4 May 2015), the VIRTIS-M infrared channel had stopped operating because of the permanent failure of the active cooler, making it impossible to further study the temporal evolution of the Anhur region. Until then, the entire part of the southern hemisphere illuminated by the Sun was observed by VIRTIS-M with a spatial resolution between 20 and 40 m per pixel. So far, CO₂ ice has been recognized only in the Anhur region discussed in this study, localized approximately

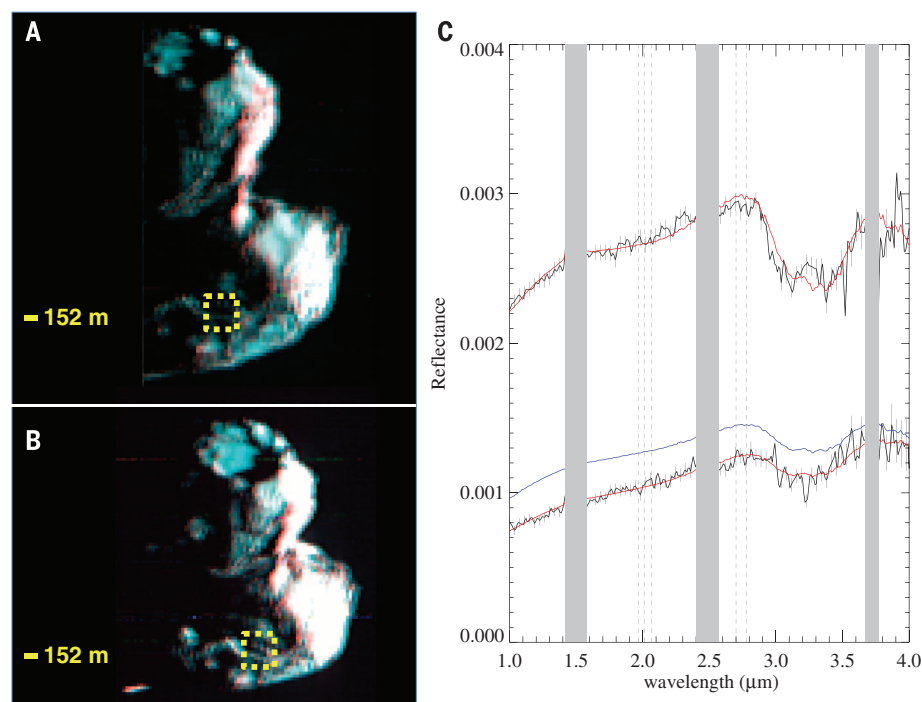


Fig. 3. The disappearance of the CO₂ surface ice by 12–13 April 2015. VIRTIS-M acquisitions (A) I1_00387470597 and (B) I1_00387561494. The position of the previously observed Anhur CO₂ ice deposit is at the center of the yellow dashed box in each panel. (C) Reflectance data from the boxed areas in the two acquisitions [bottom (A) and top (B) black lines] do not show the diagnostic triplet absorption at 2.0 μm or the bands at 2.7 and 2.78 μm (dashed vertical lines). The wide feature at 3.2 μm is the DT absorption due to organic material, the depth of which is correlated with the local reflectance level. Spectral modeling results with pure DT are shown in red. For reference, the average DT reflectance derived from (16) is shown in blue. A description of the spectral modeling is given in (12).

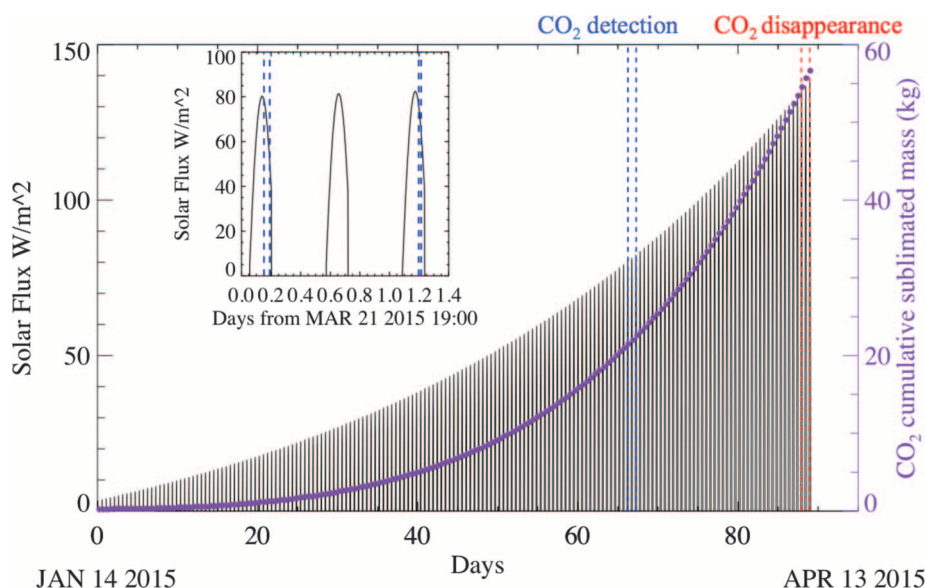


Fig. 4. Variation of solar flux reaching the Anhur CO₂ ice-rich area between 14 January and 13 April 2015. The vertical dashed lines indicate the timing of the four VIRTIS observations described in the text: CO₂ ice was detected during the first two (marked in blue; a close-up view is shown in the inset), whereas it had disappeared by the time of the last two (marked in red). The cumulative mass of sublimated CO₂ during this time frame is shown by the purple curve. All values are relative to one VIRTIS pixel area (20 by 20 m) at the time of CO₂ detection.

between a slope and a flat terrain showing a regular topography in OSIRIS images (31). The morphology and illumination conditions at this place are similar to those of many nearby areas observed by VIRTIS-M.

The presence of CO₂ ice at the surface of the nucleus thus appears to be an ephemeral occurrence, which provides clues to the emplacement mechanism. After perihelion passage, the activity of a cometary nucleus starts to decrease, with water sublimation decreasing first. Nucleus thermodynamical modeling (1) shows that a stratigraphy associated to the volatility of the major gaseous species is produced in the outer layers of 67P/CG. However, the combination of spin axis inclination and nucleus shape means that the Anhur CO₂ ice-rich area experiences a fast drop in illumination, going into permanent shadow quickly after equinox and, consequently, undergoing a rapid reduction in surface temperatures in winter to less than 80 K, whereas the interior remains warmer for a longer time because of the low thermal inertia (8, 29). Sublimation of water ice at depth is prevented, but sublimation of CO₂ ice is not; CO₂ can continue to flow from the interior to the surface, where it begins to freeze as a result of the low surface temperatures. Moving further toward the aphelion, the low surface temperatures preserve the CO₂ ice on the surface, which grows in >100-μm grains until, on the next orbit, it is exposed again to sunlight and sublimates away. This inverse temperature profile of cometary surfaces (warmer inside and cooler on the surface) going into winter after perihelion (in permanently shadowed regions) could potentially freeze other volatiles that are sublimed from the warmer interior as well. Based on the temperature of these surface areas, more volatile species such as CO and CH₄ could also be frozen until the next exposure to solar photons occurs. The same phenomenon could also explain why no water ice was seen at this site during the initial exposure to the Sun, because the water ice would have been frozen at lower depths than the CO₂ ice.

The 67P/CG nucleus shows two different temporal activity cycles respectively caused by H₂O and CO₂ ices in different regions: Whereas water ice has diurnal variability, with a surface sublimation and condensation cycle occurring in the most active areas (7), the surface condensation of CO₂ ice has a seasonal dependence. Similar processes are probably common among many Jupiter-family comets, which share with 67P/CG short revolution periods and eccentric orbits (32).

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- d’Etudes Spatiales (CNES, France), DLR (Germany), and NASA (USA). VIRTIS was built by a consortium from Italy, France, and Germany, under the scientific responsibility of INAF-IAPS, Rome, Italy, which also led the scientific operations. The VIRTIS instrument development for ESA has been funded and managed by ASI, with contributions from Observatoire de Meudon (financed by CNES) and DLR. The VIRTIS instrument industrial prime contractor was formerly Officine Galileo and is now Leonardo SpA in Campi Bisenzio, Florence, Italy. The authors thank the Rosetta Liaison Scientists, the Rosetta Science Ground Segment, and the Rosetta Mission Operations Centre for their support in planning the VIRTIS observations. T.M. acknowledges additional funding from NASA JPL, W.-H.I. from the National Science Council of Taiwan (grant 102-2112-M-008-013-MY3) and Science and Technology Development Fund of Macao Special Administrative Region (grant 017/2014/A1), and L.M. from Deutsche Forschungsgemeinschaft (grant MO 3007/1-1). The VIRTIS calibrated data are available through ESA’s Planetary Science Archive (www.cosmos.esa.int/web/psa/rosetta). This research has made use of NASA’s Astrophysics Data System.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S8
Table S1
References (33–41)

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Rosetta’s comet 67P/Churyumov-Gerasimenko sheds its dusty mantle to reveal its icy nature

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The Rosetta spacecraft has investigated comet 67P/Churyumov-Gerasimenko from large heliocentric distances to its perihelion passage and beyond. We trace the seasonal and diurnal evolution of the colors of the 67P nucleus, finding changes driven by sublimation and recondensation of water ice. The whole nucleus became relatively bluer near perihelion, as increasing activity removed the surface dust, implying that water ice is widespread underneath the surface. We identified large (1500 square meters) ice-rich patches appearing and then vanishing in about 10 days, indicating small-scale heterogeneities on the nucleus. Thin frosts sublimating in a few minutes are observed close to receding shadows, and rapid variations in color are seen on extended areas close to the terminator. These cyclic processes are widespread and lead to continuously, slightly varying surface properties.

All cometary nuclei observed to date have appeared to be dark, with only a limited amount of water ice detected in small patches (1, 2), although water is the dominant volatile observed in their coma.

The Rosetta spacecraft has been orbiting comet 67P/Churyumov-Gerasimenko since August 2014, providing the opportunity to continuously investigate its nucleus. The comet has a distinct bilobate shape and a complex morphology (3–5), with a

surface dominated by anhydrous and organic-rich refractory materials (6). Small amounts of water ice have been identified by the Visible, Infrared, and Thermal Imaging Spectrometer (VIRTIS) spectrometer in different regions (7–9), and several isolated or clustered bright spots were observed in Optical, Spectroscopic, and Infrared Remote Imaging System (OSIRIS) images and interpreted as exposures of dirty water ice (9, 10).

Here, we report on seasonal and diurnal color variations of the surface of 67P's nucleus from observations with the narrow-angle-camera (NAC) of the OSIRIS imaging system (11) onboard Rosetta, as caused by the evolution of the dust mantle and exposures of water ice.

We monitored the color evolution of the nucleus from 3.6 astronomical units (AU) to perihelion (1.24 AU) and beyond by measuring changes in the spectral slope in the 535- to 882-nm range. Comparing the spectral slopes obtained in late August 2015, shortly after perihelion passage, with those obtained in early August 2014 after phase reddening correction (12) (Fig. 1, figs. S1 to S3, and table S1) clearly indicates that the nucleus has become relatively bluer—i.e., the spectral slope has decreased as the comet approached perihelion.

Even though only the equatorial regions such as Imhotep (13) are common for both data sets (Figs. 1 and fig. S4), due to different seasonal insolation conditions, a decrease of about 30% in the mean spectral slope (12) from 3.6 AU to the perihelion passage is measured in the common areas. A decrease of the visible spectral slope was also reported from VIRTIS observations in the August to November 2014 time frame (14). In a water-ice/refractory-material mixture, regions with bluer colors indicate a higher abundance of water ice (15, 16), as demonstrated by VIRTIS observations (8, 9) and as previously observed for the 9P/Tempel 1 and 103P/Hartley 2 comets (1, 2).

In addition to the change of color, the amount of phase reddening (the increase of spectral slope with phase angle) decreased by a factor of two when the comet approached perihelion in the 2015 observations compared with those from August 2014 (12, 15), indicating a change in the physical properties of the outermost layer of the nucleus. The relatively blue color of the surface and the decreased phase reddening effect observed at small heliocentric distances suggest that the

increasing level of activity has thinned the surface dust coating, partially exposing the underlying ice-rich layer. This result is in agreement with the variation of the sublimation rate with heliocentric distance r reported by (17), considerably steeper ($\propto r^{-4.2}$) than the one obtained from modeling considering the shape of the nucleus and seasonal effects (18). This steepness can be explained by the observed thinning of the surface dust layer that facilitates the sublimation during the approach to perihelion. Observations at 2.2 AU outbound (fig. S3) show that the comet's colors redden again as the activity decreases and is no longer capable of sustained removal of dust.

The increase in water-ice visibility is observed on the whole surface, indicating that the composition in terms of dust-to-ice ratio must be similar at large scales all over the nucleus. This means that even the smooth areas commonly thought to be covered with material that fell back on the surface (18) must be water-ice rich.

We observed and monitored two bright patches, of about 1500 m² each (table S2), located in a smooth area of the Anhur/Bes (13) regions (Fig. 2 and fig. S4), on images acquired on 27 April to 2 May 2015. One of the two patches, patch B in Fig. 2, was still present in an image acquired on 7 May, yielding a survival time of at least 5 to 10 days. These features are considerably larger than the meter-scale bright spots previously detected on the 67P comet (10). The VIRTIS spectrometer detected the presence of CO₂ ice in region A on 21 to 23 March 2015 that had sublimated in less than 3 weeks (19). On 12 April, the bright patches were not present yet, but these regions were spectrally bluer than the surrounding ones, indicating a higher water-ice abundance (Fig. 2). On 27 April and 2 May, the bright patches were clearly visible and showed a relatively flat spectrum, with a maximum reflectance four to six times as bright as the surrounding regions. A water-ice surface abundance of ~25% linearly mixed with the comet

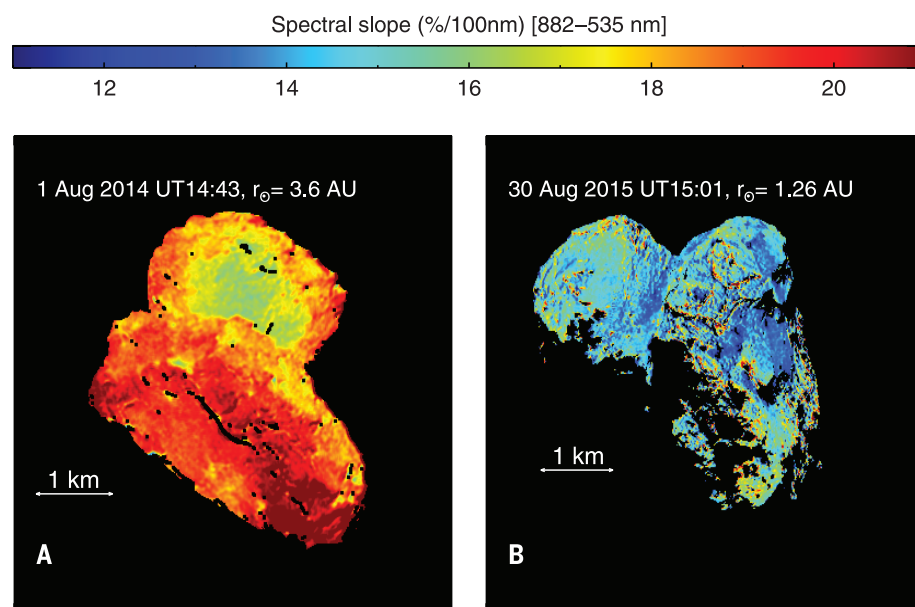


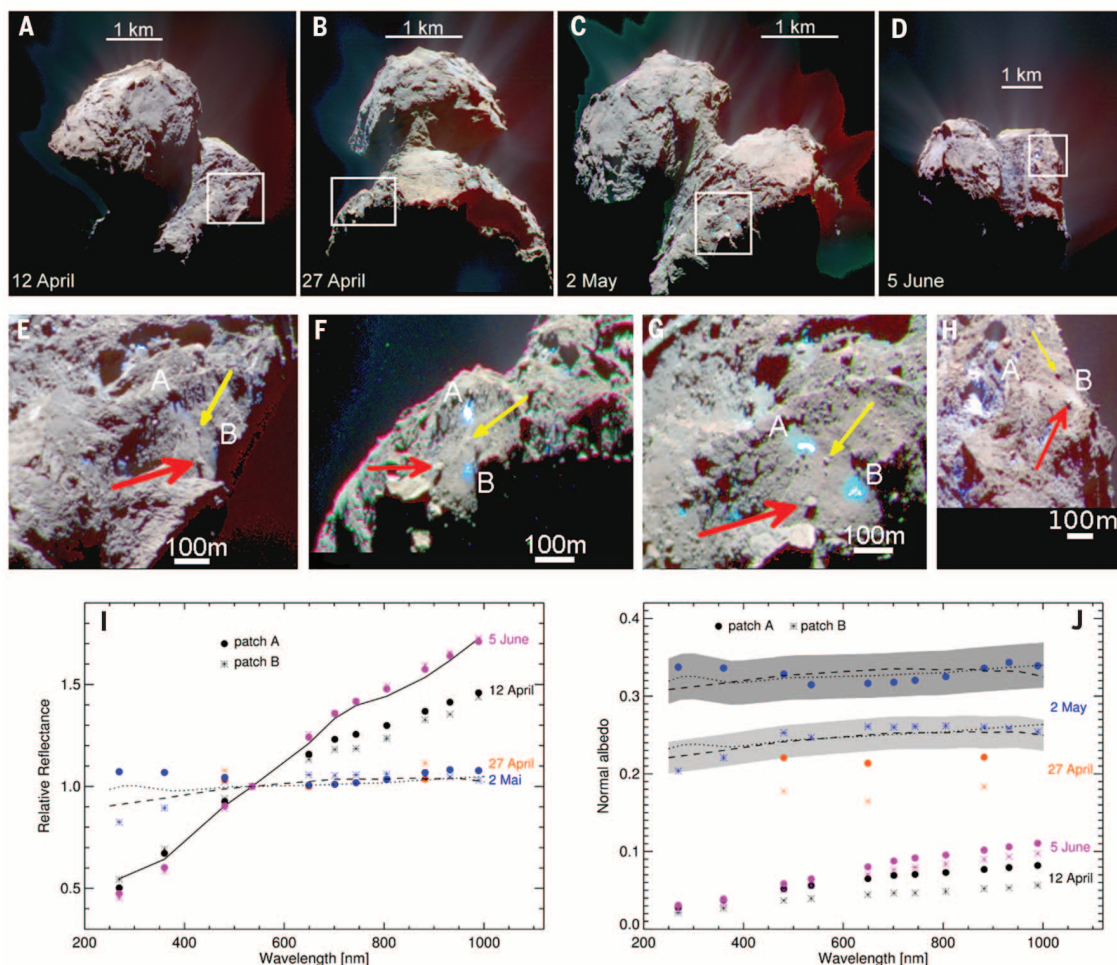
Fig. 1. Spectral slope evolution with heliocentric distance. The August 2014 image was corrected for the phase reddening from 10° to 70° using the coefficients published in (15) to match the viewing geometry on the right. See fig. S4 for the morphological regions context.

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Fig. 2. Anhur/Bes ice-rich patches.

Composite images (882, 649, and 480 nm filters) showing the appearance of the bright patches in the Anhur/Bes regions (**A** to **D**), and associated zooms (**E** to **H**); the arrows indicate two common boulders. The reflectance relative to 535 nm and the normal albedo are represented in (**I**) and (**J**). The black line represents the mean spectrum of the comet from a region close to the patches. Dashed and dotted lines (**J**) show the best-fit spectral models to the patches (associated uncertainty shown in gray) produced by the linear mixture of the comet dark terrain (12) enriched with $21 \pm 3\%$ of water ice (dashed line) or $23 \pm 3\%$ of water frost (dotted line) for patch B, and with $29 \pm 3\%$ of water ice (dashed line) or $32 \pm 3\%$ of water frost (dotted line) for patch A.



dark terrain is needed to match the patches' reflectance (12). Subsequent images covering the Anhur region were acquired on 4 to 5 June, revealing that the water ice had fully sublimated from the surface, leaving a layer spectrally indistinguishable from the average nucleus (Fig. 2).

We compute the sublimation rate for this period by applying the thermal model described in (18) for both intimate and areal mixtures of refractory material and ice (12). The estimated ice loss rate ranged from 1.4 to $2.5 \text{ kg day}^{-1} \text{ m}^{-2}$ for the intimate mixture and from 0.14 to $0.38 \text{ kg day}^{-1} \text{ m}^{-2}$ for the areal mixture cases. By noting that the permanence of the ice patches was about 10 days, we estimate a solid-ice equivalent thickness between 1.5 and 27 mm for the ice patches. The actual thickness of this layer will be up to a factor of 10 higher due to porosity of the dust/ice mixture.

The appearance and disappearance of water-ice patches shows that the level of activity is varying on time scales that are short compared with seasonal changes of the illumination. These ice-rich patches indicate a variation of the water-ice content in the uppermost layers, pointing to local compositional heterogeneities with scales of 10s of meters on 67P's nucleus. The huge width-to-depth ratio observed in the ice patches would suggest a near-surface solar-driven process being

responsible for enhancement of local ice abundance, resulting from the recondensation of volatiles and sintering of the subsurface material during previous perihelion passages. Laboratory results from Kometen Simulation (KOSI) experiments had shown that a considerable fraction of sublimating ice can be redeposited instead of being released through the dust mantle (20). Numerical simulations show that a hardened layer may form beneath a fine dust mantle (21), a hard layer that was detected by the Philae lander in the Abydos site (22). The composition of the icy patches may be representative of the comet's near surface. Occasional local removal of the overlying mantling material could expose the underlying layer, leading to an icy surface for limited periods of time.

Approaching perihelion, the nucleus has shown considerable diurnal color variations on extended areas and the occurrence of water frost close to morning shadows. This is evident on the Imhotep region, where morphological changes were observed (23). Areas just emerging from the shadows are spectrally bluer than their surroundings (Fig. 3A), while 40 min later, once fully illuminated, their spectral slope has increased (Fig. 3B). This phenomenon, observed during other color sequences acquired in June and July 2015, and seen at dawn on different areas on both lobes of the

comet (12) (fig. S5 and movie S1), is periodic. We interpret the relatively blue surface at dawn as the presence of additional water frost that condensed during the previous night.

We also observe the presence of fronts of bright material in the illuminated regions close to rapidly traveling shadows cast by local topography (Fig. 3). A water-rich fringe near shadows at the Hapi/Hathor transition was also observed by VIRTIS (7). The bright fronts move with the shadows. Modeling of the illumination (fig. S6 and movie S2) shows that the extent of these bright features directly correlates with the shadow travel speed, being wider where the shadow speed is faster and narrower where the shadow speed is slower.

These fronts are about six times as bright as the mean comet reflectance. Their spectrum is globally flat with a flux enhancement in the ultraviolet (Fig. 4 and fig. S7), similar to that observed on blue regions of comet Tempel 1 (1). An abundance of about 17% water frost linearly mixed with the comet dark terrain is needed to match the reflectance of the bright fronts (12). We interpret these bright features as surface frost, formed when water vapor released from the subsurface recondenses after sunset, that rapidly sublimates when exposed to the Sun. Molecules in the inner

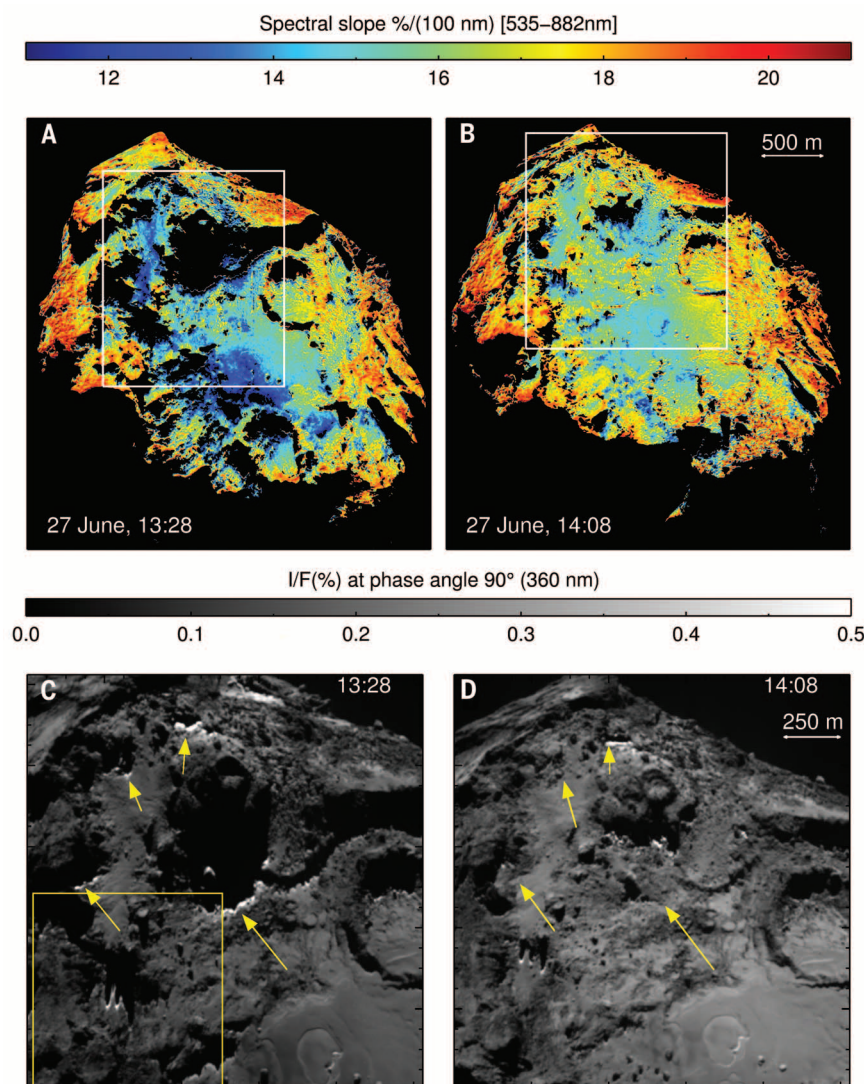


Fig. 3. Spectral slope changes and frost sublimation. (A and B) Spectral slope maps of Imhotep region taken 40 min apart. The Sun is toward the top. (C and D) Zoom in radiance factor (I/F) of the regions indicated by the white rectangle on (A) and (B) showing morning frosts (evidenced by the yellow arrows), disappearing and moving with shadows. The yellow rectangular region in (C) indicates the area where the same frost structures are seen 2 weeks later and analyzed in Fig. 4.

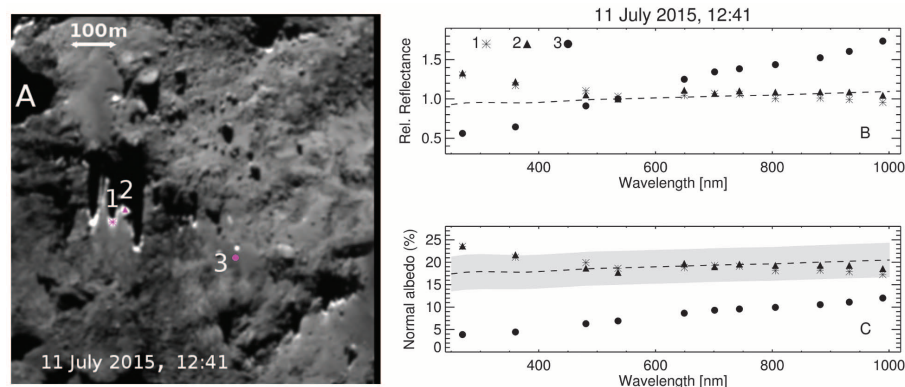


Fig. 4. Reflectance of frost fronts. (A) Bright fronts seen close to shadows on the Imhotep region (see Fig. 3 for the context). (B and C) Reflectance normalized at 535 nm and normal albedo of three selected regions [magenta points in (A)]. The dashed line represents the best-fit model, with uncertainties in gray, including $17 \pm 4\%$ of water frost linearly mixed to the comet dark terrain.

coma may also be back-scattered to the nucleus surface and recondense, contributing to the frost formation (24, 25). From the shadow travel speed and the extent of the frost fronts, we calculate an ice permanence time of about 3 min (12). From thermal modeling using an intimate mixture, we estimate a water-ice sublimation rate of about $4 \text{ g m}^{-2} \text{ min}^{-1}$, from which we infer a total ice content of about 12 g m^{-2} for the frost layer, which is extremely thin (thickness ~ 10 to $15 \text{ }\mu\text{m}$ of equivalent solid ice) (table S3). If we assume an areal mixing, the sublimation rate and total ice content are lower by a factor of 8. In the absence of direct temperature measurements of the frost, it is impossible to discriminate whether areal or intimate mixture represents the correct thermal model (1). In either case, the exposed ice is depleted on short time scales. In the case of geographical mixing, however, sublimation rates at the time of the observations would be so low that a frost permanence time of 3 min would imply a frost layer on the order of $\sim 1 \text{ }\mu\text{m}$ solid-ice equivalent. Because it is questionable whether such a thin layer would be optically thick, it appears more likely that the frost patches are intimately mixed with the refractory material. Similar to the observed diurnal color variations, the recondensation of frosts is also a periodic phenomenon that takes place close to topographic shadows, as indicated in Figs. 3 and 4.

The long-term observations of 67P provide information on the composition of the outermost layers of the nucleus. They reveal that ice is abundant just beneath the surface on the whole nucleus, which, most of the time, is covered by a thin layer of dust. Hence, the apparent surface composition is globally dominated by anhydrous refractory materials (6). The increasing cometary activity while approaching the Sun progressively thins the dust surface layer, partially exposing the ice-rich subsurface, yielding nucleus's colors that are bluer relative to those at large heliocentric distances.

OSIRIS observations show that mixtures having high abundance (up to $\sim 30\%$) of water ice are occasionally locally present on the nucleus and that the lifetime of exposed ice is short, on the order of a few minutes to a few days. However, no dust-free ice patches were observed even during the peak of activity near perihelion, indicating that water ice and dust are well mixed within the resolution limit of the images ($\sim 2 \text{ m pixel}^{-1}$). Most of the bright features are observable only at high spatial resolution and under particular insolation conditions—i.e., close to the morning shadows. Similar phenomena presumably take place on other comets, explaining why cometary nuclei are so dark even if they have important water ice abundance. The extended bright patches observed in the Anhur/Bes regions indicate a local enrichment of water ice, pointing to compositional heterogeneities in the uppermost layers of comet 67P.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6319/1566/suppl/DC1
Materials and Methods

Figs. S1 to S7

Tables S1 to S3

Movies S1 and S2

References (26–31)

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CATALYSIS

Selective oxidative dehydrogenation of propane to propene using boron nitride catalysts

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The exothermic oxidative dehydrogenation of propane reaction to generate propene has the potential to be a game-changing technology in the chemical industry. However, even after decades of research, selectivity to propene remains too low to be commercially attractive because of overoxidation of propene to thermodynamically favored CO₂. Here, we report that hexagonal boron nitride and boron nitride nanotubes exhibit unique and hitherto unanticipated catalytic properties, resulting in great selectivity to olefins. As an example, at 14% propane conversion, we obtain selectivity of 79% propene and 12% ethene, another desired alkene. Based on catalytic experiments, spectroscopic insights, and ab initio modeling, we put forward a mechanistic hypothesis in which oxygen-terminated armchair boron nitride edges are proposed to be the catalytic active sites.

Selective oxidation technology plays a pivotal role in the modern chemical industry (1). The scientific challenge for partial oxidations is the prevention of consecutive overoxidation of the desired product, which is often more reactive than the parent substrate. The oxidative dehydrogenation of propane (ODHP) is a contemporary example of such a challenging reaction. Although propene is conventionally produced via steam cracking of large hydrocarbons in naphtha, the more recent trend to use shale gas as feeds to steam cracker units greatly increases the availability of low-cost ethene

but also results in a gap between demand and supply of propene, as well as other higher olefins (2, 3). This disparity motivates the exploration of “on-purpose” propene technologies. Even though nonoxidative propane dehydrogenation is the emerging technology used today, ODHP has the potential to improve reaction efficiency over its nonoxidative counterpart because of favorable thermodynamics (exothermic and lower reaction temperatures) and enhanced catalyst stability (prevention of coke deposition on the catalyst surface). It is estimated that the potential energy savings for moving to ODHP would be ~45% of the energy consumption (4, 5). This gain in reaction efficiency, coupled with the facts that demand for propene is around 100,000 kt and its production consumes around 1 quad of energy, illustrates the potential impact of ODHP if it could be implemented on a large scale. The key

scientific challenge that must be overcome remains, however, the prevention of the facile overoxidation of propene product into more thermodynamically stable CO and CO₂ (CO_x).

To date, supported vanadium oxide catalysts show the most promising activity for ODHP, owing to the favorable redox properties of the active vanadium sites (6, 7). However, even after decades of research, propene selectivity remains too low, even at moderate propane conversion. As an example, at 10% propane conversion, the propene selectivity typically drops to less than 60% for such conventional catalysts. The lack of kinetic control identifies the need for the discovery of alternative materials with the ability to better control this partial oxidation (8).

Here, we present both hexagonal boron nitride (h-BN) and boron nitride nanotubes (BNNTs) as metal-free materials able to catalyze the ODHP reaction. Although graphene and fullerene materials are emerging as catalysts for partial alkane oxidations (9–11), BN materials, one of the “inorganic analogs” of graphene, have yet to be explored for their own catalytic activity. A supported vanadia on silica catalyst (V/SiO₂) was used in this work to make direct comparisons with the catalytic performance of BN. These materials were loaded into a quartz tube reactor heated between 460 and 500°C under flowing propane, oxygen, and nitrogen as an inert carrier gas. Reaction parameters—such as temperature, catalyst mass, total gas flow-rate, and partial pressures of propane (P_{C₃H₈}) and oxygen (P_{O₂})—were varied to observe changes to product distributions by sampling the reactor exhaust stream via online gas chromatography and mass spectrometry. Gas contact time with the catalyst is represented in this work as the inverse weight-hour-space-velocity {WHSV⁻¹ [kg-catalyst s (mol C₃H₈)⁻¹]}, which was varied primarily by altering the total gas flow rate.

Use of BN materials results in extraordinary selectivity to propene, among the highest reported under ODHP conditions. For instance,

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h-BN afforded 79% selectivity to propene at 14% propane conversion (Fig. 1A). Meanwhile, the traditional V/SiO₂ allows for a modest 61% propene selectivity at only 9% propane conversion (22). The selectivities obtained by using state-of-the-art ODHP catalysts (11, 13–19) are compared in Fig. 1A. The decrease in propene selectivity with increasing propane conversion is indicative of the facile overoxidation of propene to CO_x. Comparisons between key reaction parameters of the referenced catalysts are included in table S1.

The entire product distribution further distinguishes BN materials from supported vanadia catalysts (Fig. 1B). When the supported vanadia catalyst is used, the main by-products are CO_x, which account for 33% of total product selectivity at 9% propane conversion. Conversely, when BN materials are used, the main by-product is ethene, a highly valuable olefin itself, rather than CO_x. The combined propene and ethene selectivity is 91% at 14% propane conversion using h-BN (fig. S1). We furthermore verified that the catalytic activity of the BN material remains stable for at least 32 hours on stream (fig. S2), validating the catalyst stability. Transmission electron microscopy images of the h-BN material before and after the ODHP reaction did not reveal any changes to the morphology of the material (fig. S3). Carbon balances for all reactions close within >98%.

The analogous product distributions for both h-BN and BNNTs suggest a similar reaction mechanism for these BN materials. However, BNNTs exhibit a rate of propane consumption (mol C₃H₈ kg-cat⁻¹ s⁻¹) more than one order of magnitude as high as that observed with h-BN (fig. S4). The higher activity of BNNTs at least partially reflects the higher surface area of BNNTs relative to h-BN (BNNT, 97 ± 5 m² g⁻¹ versus h-BN, 16 ± 1 m² g⁻¹) (20); however, the rate of propane consumption is more than three times as high with BNNT as with h-BN when normalized for surface area (BNNT: 3.6 × 10⁻⁷ mol C₃H₈ s⁻¹ m⁻² versus h-BN: 1.1 × 10⁻⁷ mol C₃H₈ s⁻¹ m⁻²). This high reactivity and selectivity with BNNTs results in a substantial enhancement in the observed propene

productivity (kg-C₃H₆ kg-cat⁻¹ hr⁻¹) (Fig. 1C), comparable to values deemed attractive for commercial implementation of this on-purpose propene technology (15, 21).

Further kinetic insights were obtained by studying the influence of reactant concentrations [partial pressure of O₂ and C₃H₈ (*P*_{O₂} and *P*_{C₃H₈}, respectively)] on the reaction rate. The inclusion of oxygen as a reactant is required for propane conversion using BN materials. The rate of propane consumption using h-BN indicates oxygen activation on the BN surface (Fig. 2A) and second-order dependence with respect to *P*_{C₃H₈} (Fig. 2B). This kinetic behavior clearly distinguishes BN from traditional supported vanadia catalysts, which follow a Mars-van Krevelen mechanism (rate-determining substrate oxidation followed by fast reoxidation of the surface by oxygen) that typically leads to zero-order rate dependence with respect to *P*_{O₂} and first-order rate dependence in *P*_{C₃H₈} (22).

It is surprising that BN, a material known for its high stability under oxidative conditions (23, 24), is catalytically active at all. So far it has been explored for its unique electronic, thermoelectric, and mechanical properties (25–28). The combination of the interesting observations outlined in this work (i.e., improved selectivity to olefins and different reaction kinetics) points toward a fundamentally different reaction mechanism in conjunction with a different active site compared with other, previously studied and better understood catalysts for this reaction. Indeed, BN samples from various suppliers and containing different impurities (fig. S5) show almost identical catalytic performance (figs. S6 and S7), which indicates that said metal impurities in the material are unlikely to play a significant role.

To better understand the catalytically active parts of the material and to provide evidence for oxygen functionalization of the BN surface when exposed to ODHP reaction conditions, we used a combination of x-ray photoelectron spectroscopy (XPS), attenuated total reflectance-infrared (ATR-IR), and diffuse reflectance infra-

red Fourier transform spectroscopy (DRIFTS) measurements to characterize the material before (“fresh”) and after (“spent”) exposure to the ODHP reaction mixture. As a first step, we used XPS to monitor the surface concentrations of boron, nitrogen, and oxygen of h-BN (table S1) and observed an increase in the amount of surface oxygen in spent h-BN material, signaling oxygen functionalization. The same increase in surface oxygen content was not observed for h-BN exposed only to air at 490°C; this indicated that the hydrocarbon must be present for oxygen to functionalize the surface of h-BN. This observation was corroborated with ATR-IR measurements, which showed the emergence of a broad feature around 3200 cm⁻¹ and a sharp signal at 1190 cm⁻¹ for spent h-BN (fig. S8A), assigned to OH-stretching and B–O stretching vibrations, respectively (29, 30). These spectroscopic features were absent with fresh h-BN, as well as h-BN treated only under air at 490°C. Using DRIFTS, we saw more-resolved features appear at 3420 and 3250 cm⁻¹ with spent h-BN (fig. S8B). BNNTs show a similar behavior, only with considerable intensity in the given spectral range already present for the fresh samples and a corresponding increased intensity for the spent materials (fig. S9).

The appearance and change of the different spectroscopic features furthermore indicates that a site is created by exposure of the material to propane and oxygen and that, in line with the higher intensity of this feature, a larger number of these sites are present in the BNNT sample, and the exposure to both propane and oxygen leads to the creation of those sites. To elucidate the origin of these vibrations, we performed *ab initio* density functional theory (DFT) calculations using the Vienna *ab initio* simulation package (VASP) (31, 32). All computational details are given in the supplementary materials. We focused on a single BN sheet and calculated vibrational frequencies for a set of OH-terminated zigzag and armchair edges. All structures and corresponding vibrational frequencies >3000 cm⁻¹ are given in fig. S10. When comparing the modeled

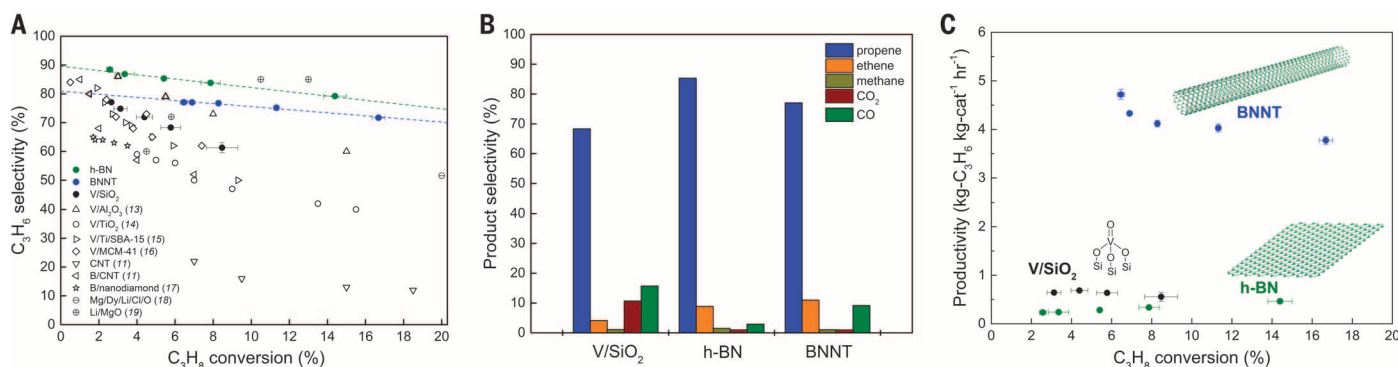


Fig. 1. Selectivity to propene and comparisons of product selectivity. (A) Selectivity to propene plotted against propane conversion for ODHP, comparing previously reported data from representative catalysts to hexagonal boron nitride (h-BN) and boron nitride nanotubes (BNNTs). Open shapes indicate data from other works, cited within the figure. (B) Comparisons of product selectivity between V/SiO₂ (*X*_{C₃H₈} = 5.8%); h-BN (*X*_{C₃H₈} = 5.4%); and BNNTs (*X*_{C₃H₈} = 6.5%). Product selectivity is represented by colored bars. (C) Comparisons of propene productivity plotted as a function of C₃H₈ conversion between V/SiO₂, h-BN, and BNNT. V/SiO₂: 5 to 15 kg-cat s mol C₃H₈⁻¹; h-BN: 15 to 40; BNNT: 2 to 5; *T* = 490°C, *P*_{O₂} = 0.15 atm, *P*_{C₃H₈} = 0.3 atm.

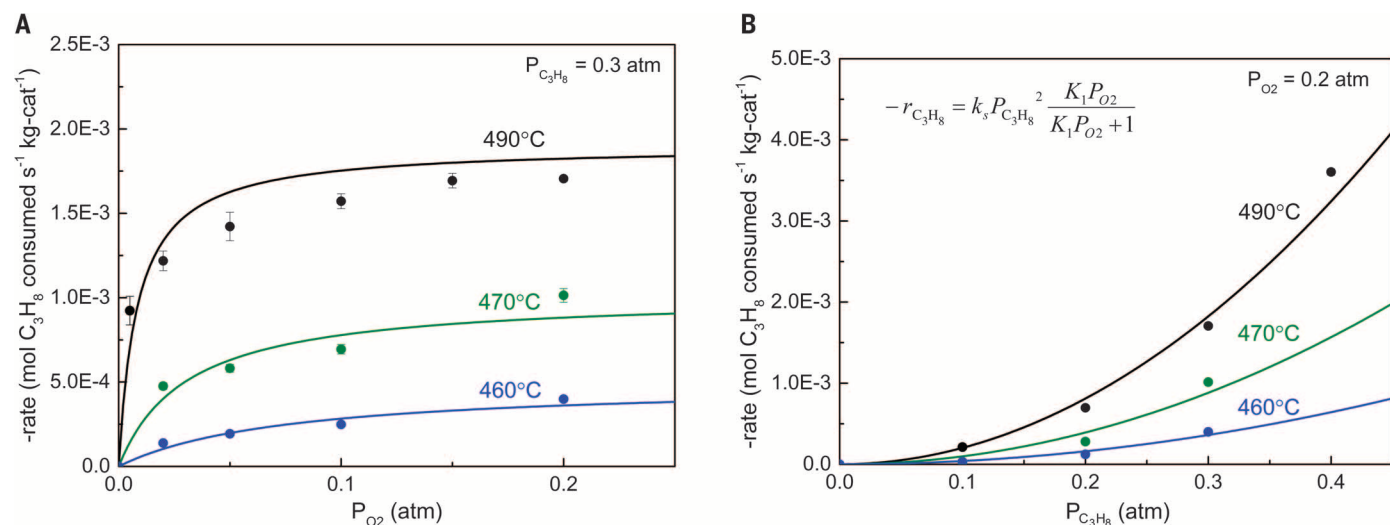


Fig. 2. Rates of propane consumption. Rates of propane consumption using h-BN as a function of (A) P_{O_2} ($P_{\text{C}_3\text{H}_8}$ constant at 0.3 atm) and (B) $P_{\text{C}_3\text{H}_8}$ (P_{O_2} constant at 0.2 atm) fit with Eley-Rideal kinetics showing O_2 adsorption and second-order dependence with respect to $P_{\text{C}_3\text{H}_8}$. Solid lines are a least-squares fit that takes into account all experimental data points at each respective temperature by using the displayed rate law.

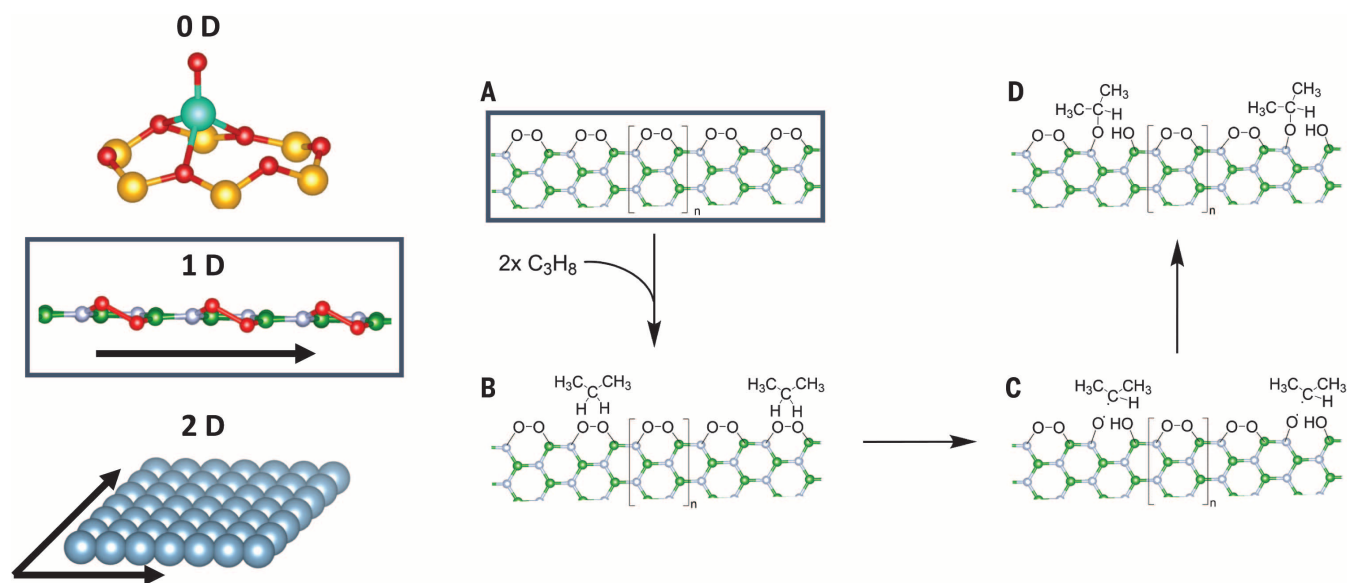


Fig. 3. Proposed radical rebound mechanism for hydrogen atom abstraction from propane to the O-terminated armchair BN edge site. (A to D) Our model suggests that this O-terminated edge site (one-dimensional, 1D) may lead to the observed favorable olefin selectivity. This is drastically different from a 2D site (e.g., V/SiO₂), which most likely creates a reactive propyl radical (34), or a 2D site [e.g., Cu(111) surface] where diffusion of adsorbates is unrestricted (35), both prone to overoxidation.

wavenumbers to the experimental measurements, we find that especially the armchair edge leads to features in the studied range, the feature around 3420 cm^{-1} corresponding to a single OH-stretch (displayed in movie S1) and the feature around 3250 cm^{-1} associated with a concerted stretching vibration (displayed in movie S2).

From the combination of catalytic activity, spectroscopic data, and DFT calculations and in line with the observed oxygen-dependence of the kinetics, we therefore propose that an oxygen molecule bonded to one B and one N [an oxygen-terminated armchair edge of BN ($>\text{B}-\text{O}-\text{N}<$)]

acts as active site for the ODHP reaction. This site shows similarities to work in the semiconductor literature focusing on oxygen-terminated armchair edges of BN (28), as well as the proposed active sites of graphene and fullerene materials for similar oxidations (10, 11). These $>\text{B}-\text{O}-\text{N}<$ sites can be viewed as inorganic peroxide species able to perform oxidation reactions. Starting from our modeled active site (namely, $>\text{B}-\text{O}-\text{N}<$), we then calculated key intermediates for the ODHP reaction. We suggest that the dehydrogenation initiates by the abstraction of a hydrogen atom from a second-

ary carbon of propane by the $>\text{B}-\text{O}-\text{N}<$ sites, breaking the O-O bond while forming a B-OH species and one nitroxyl radical (Fig. 3C). This reaction is reminiscent of an alkyl hydroperoxide reaction proposed earlier to explain the formation of radicals in homogeneous autoxidation systems ($\text{RO}-\text{OH} + \text{H}-\text{R}' \rightarrow \text{RO}' + \text{HOH} + \text{R}'$) (33). However, in the case of the surface-catalyzed reaction, we anticipate a rebound step where the secondary propyl radical reacts rapidly with the nitroxyl radical, forming a surface-stabilized propyl intermediate [namely, $>\text{N}-\text{O}-\text{CH}(\text{CH}_3)_2$] (Fig. 3D).

We consider that the observed high olefin selectivity may largely be due to the stabilization of the propyl radical by the nitroxyl radical site. Indeed, the one-dimensional (1D) nature of this edge avoids the creation of a highly reactive propyl radical (typical of 0D single-site catalysts) (34), and also the overoxidation of the adsorbed species (typical of 2D surface catalysts) is suppressed (35). The proposed intermediate structures and energies of the overall catalytic cycle are included in fig. S11. We envision that a second abstraction of a hydrogen atom from a primary carbon follows another radical rebound, and creates a di-propoxyl intermediate. Desorption of propene and the reorganization of hydrogen atoms along the edge form water as a side product. The desorption of water is followed by oxygen addition to regenerate the $>\text{B}-\text{O}-\text{O}-\text{N}<$ active site. Similar surface reorganization of hydroxyl groups in the presence of oxygen was proposed for related carbon nanofilament catalysts for oxidative dehydrogenation of ethylbenzene to form styrene (36). All steps in this process, except for desorption of propene, are exothermic. The second-order rate dependence with respect to Pc_3H_8 suggests that two propane molecules are required to generate two molecules of water, in line with the overall stoichiometry of the reaction. The desorption of these water molecules forms BN edge vacancies that allow for unique O_2 activation, which explains the influence that the surface coverage of adsorbed oxygen has on the rate of propane consumption.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6319/1570/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S14
Tables S1 and S2
Movies S1 and S2
References (37–40)

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QUANTUM ELECTRONICS

Suppressing relaxation in superconducting qubits by quasiparticle pumping

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Dynamical error suppression techniques are commonly used to improve coherence in quantum systems. They reduce dephasing errors by applying control pulses designed to reverse erroneous coherent evolution driven by environmental noise. However, such methods cannot correct for irreversible processes such as energy relaxation. We investigate a complementary, stochastic approach to reducing errors: Instead of deterministically reversing the unwanted qubit evolution, we use control pulses to shape the noise environment dynamically. In the context of superconducting qubits, we implement a pumping sequence to reduce the number of unpaired electrons (quasiparticles) in close proximity to the device. A 70% reduction in the quasiparticle density results in a threefold enhancement in qubit relaxation times and a comparable reduction in coherence variability.

Since Hahn's invention of the spin echo in 1950 (1), coherent control techniques have been crucial tools for reducing errors, improving control fidelity, performing noise spectroscopy, and generally extending coherence in both natural and artificial spin systems. All of these methods are similar: They correct for dephasing errors by reversing unintended phase accumulations due to a noisy environment through the application of a sequence of control pulses, thereby improving the dephasing time T_2 . However, such coherent control techniques cannot correct for irreversible processes that reduce the relaxation time T_1 , where energy is lost to the environment. Improving T_1 requires reducing the coupling between the spin system and its noisy environment, reducing the noise in the environment itself (2), or implementing full

quantum error correction. We demonstrate a pumping sequence that dynamically reduces the noise in the environment and improves T_1 of a superconducting qubit through an irreversible

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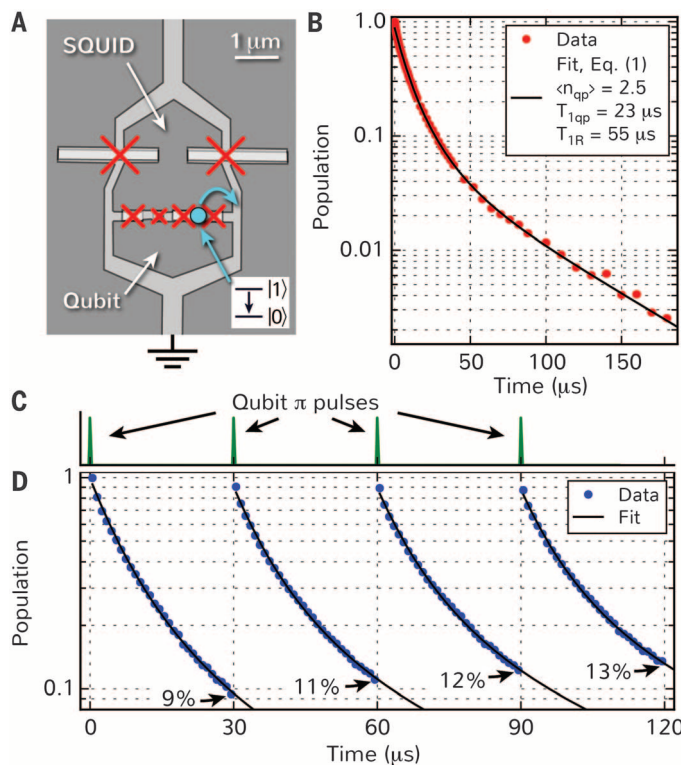
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Fig. 1. Nonexponential decay in a superconducting flux qubit.

(A) Schematic drawing of device A, consisting of a flux qubit (lower loop) coupled to a dc superconducting quantum interference device (SQUID) for qubit readout (outer loop). Red crosses mark the position of the Josephson junctions. Qubit relaxation is induced by quasiparticles tunneling across the qubit junctions, as illustrated by the blue circle.

(B) Qubit decay, as measured by applying a π pulse and delaying the qubit readout. The decay is clearly nonexponential, with the solid line showing a fit to the decay function expected from quasiparticle tunneling [Eq. 1 in the main text].

(C) Pulse sequence for pumping quasiparticles away from the qubit junctions, consisting of multiple qubit π pulses separated by a fixed period ΔT . (D) Average qubit population during the pumping sequence, measured by repeating the experiment over 40,000 trials. The remaining population after each pulse interval steadily increases, demonstrating that the qubit decay becomes progressively slower. The solid lines are fits to Eq. 1, with $\langle n_{qp} \rangle$ decreasing as {2.4, 1.9, 1.7, 1.6} from the first to the last decay.



pumping process. The sequence contains the same type of control pulses common to all dynamical-decoupling sequences, but rather than coherently and deterministically controlling the qubit time evolution, the sequence is designed to shape the noise stochastically via inelastic energy exchange with the environment. Similar methods have been used to extend T_2 of spin qubits by dynamic nuclear polarization (3), and irreversible control techniques are commonly used to prepare systems into well-defined quantum states through optical pumping (4, 5) and sideband cooling (6). However, outside of quantum error correction, to our knowledge no dynamic enhancement of T_1 has been previously reported.

We implement the pumping sequence in a superconducting flux qubit, with the aim of reducing the population of unpaired electrons or quasiparticles in close vicinity to the device. When a superconducting circuit is cooled well below its critical temperature, the quasiparticle density via Bardeen-Cooper-Schrieffer theory is expected to be exponentially suppressed, but a number of experimental groups have reported higher-than-expected values in a wide variety of systems (7–10). Although the reasons for the enhanced quasiparticle population and the mechanism behind quasiparticle generation are not fully understood, their presence has a number

of adverse effects on the qubit performance, causing relaxation (11–16), dephasing (17–19), excess excited-state population (20), and temporal variations in qubit parameters (21–24). Moreover, quasiparticles are predicted to be a major obstacle for realizing Majorana qubits in semiconductor nanowires (25). Our results provide an *in situ* technique for removing quasiparticles, especially in conjunction with recent experiments showing that vortices in superconducting electrodes can act as quasiparticles traps, thus keeping the quasiparticles away from the Josephson junctions where they may contribute to qubit relaxation (22, 26–28).

We characterize and quantify the quasiparticle population by measuring qubit relaxation. Generally, the relaxation rate is given by a sum of contributions from many different decay channels. Quasiparticles contribute to the relaxation in a process whereby the qubit releases its energy to a quasiparticle tunneling across one of the Josephson junctions (Fig. 1A). Because of the small number of quasiparticles typically present in the device, fluctuations in the quasiparticle population lead to large temporal variations in the qubit decay rate. As a consequence, if the number of quasiparticles changes between trials while one repeats an experiment to determine the average qubit polarization, the time-domain

decay no longer behaves as a single exponential but rather takes the following form (27) [see also (29), section S2]

$$p(t) = e^{\langle n_{qp} \rangle (\exp[-t/\tilde{T}_{1qp}] - 1)} e^{-t/T_{1R}} \quad (1)$$

Here, $\langle n_{qp} \rangle$ is the average quasiparticle population in the qubit region during the experiment; t is the time after qubit excitation; $\tilde{T}_{1qp}$ is the relaxation time induced by one quasiparticle; and T_{1R} is the residual relaxation time from other decay channels such as flux noise, Purcell decay, or dielectric losses. Because only quasiparticles are responsible for the nonexponential decay, Eq. 1 provides a direct method for separating out quasiparticle contributions from other relaxation channels.

The experiments are performed using two different flux qubits. Device A is a traditional flux qubit with switching-current readout using a dc superconducting quantum interference device (SQUID), whereas device B is a capacitively shunted (C-shunt) flux qubit (24). Qubit A was operated at a frequency of 5.4 GHz, whereas qubit B was operated at 4.7 GHz [for more information on qubit parameters, see sections S1 and S6 in (29)]. Figure 1B shows the measured relaxation of qubit A, together with a fit to Eq. 1. The decay is clearly nonexponential: The fast initial decay due to quasiparticle fluctuations is followed by a slower, constant decay attributable to residual relaxation channels. From the fit, we find an average quasiparticle population $\langle n_{qp} \rangle = 2.5$, with $\tilde{T}_{1qp} = 23 \mu s$ and $T_{1R} = 55 \mu s$. We have also measured the qubit decay as a function of flux and temperature to further validate its sensitivity to quasiparticles [sections S3 and S4 in (29)].

The same mechanism that leads to qubit relaxation also provides an opportunity for reducing the quasiparticle population. When the qubit relaxes through a quasiparticle tunneling event, the quasiparticle both tunnels to a different island and acquires an energy $\hbar\omega_0$ from the qubit ($\omega_0/2\pi$ is the qubit frequency and $\hbar$ is Planck's constant h divided by 2π). The increase in energy leads to a higher quasiparticle velocity (at a constant mean free path) so that a quasiparticle can move more quickly away from the regions close to the qubit junctions where it may cause qubit relaxation. The situation is depicted in Fig. 1A, where the quasiparticle tunneling out from the section of the qubit loop containing the junctions may diffuse away toward the normal-metal ground electrode. We make use of this mechanism by applying a pulse sequence (Fig. 1C) consisting of several qubit π pulses separated by a fixed period (in this case, $\Delta T = 30 \mu s$). The first π pulse excites the qubit into state $|1\rangle$ and, during the subsequent waiting time, it has some probability of relaxing to the ground state. Because $\tilde{T}_{1qp} < T_{1R}$, this most likely occurs through a quasiparticle tunneling event, which transfers a quasiparticle across a junction, increases the quasiparticle energy, and thereby enhances its diffusion rate. The process is stochastic and may transfer quasiparticle in any direction, but by repeating the sequence we expect to pump

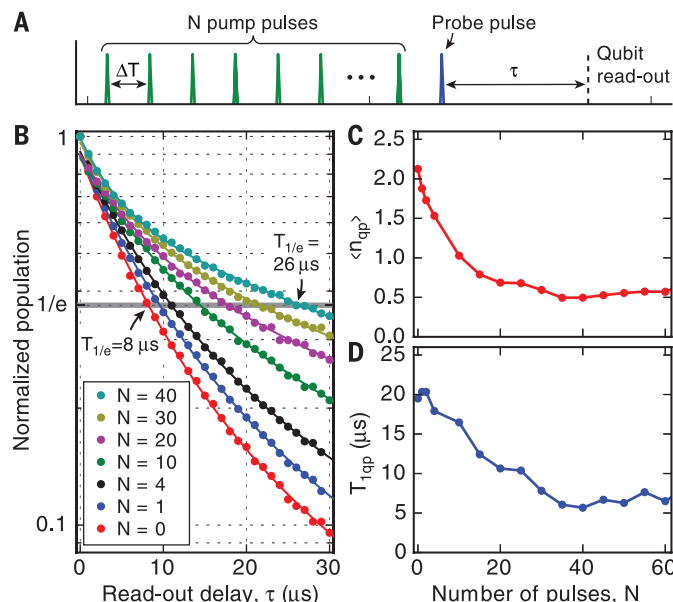
quasiparticles away from the qubit junctions. The measured average qubit polarization during the pumping sequence (Fig. 1D) starts with the qubit in the ground state, the first π pulse brings the qubit to $|1\rangle$, and during the following waiting period the qubit relaxes back to an average polarization of 9%. The second π pulse inverts the polarization to 91%, and the qubit starts decaying again. However, at the end of the second waiting period the remaining polarization is 11%, demonstrating that the decay is slower during the second interval. The third and fourth π pulses further retard the decay, yielding a remaining polarization of 12 and 13%, respectively. Note that the excess population can be removed by using single-shot readout techniques to reset the qubit state after the pumping sequence ends (30).

We quantify the reduction in qubit decay by extending the pump sequence to contain more π pulses and fitting the decay to Eq. 1. The measured qubit decay, using up to 40 pumping pulses (Fig. 2), demonstrates a more than threefold enhancement in qubit decay time compared with the bare decay (the decay time is defined as the time $T_{1/e}$ it takes for the signal to decay by a factor of $1/e$). The solid lines in Fig. 2B are fits to Eq. 1; Fig. 2, C and D, show the resulting fitting parameters $\langle n_{qp} \rangle$ and $\tilde{T}_{1qp}$ as a function of the number of pumping pulses. The average quasiparticle population drops from $\langle n_{qp} \rangle = 2.2$ to $\langle n_{qp} \rangle \sim 0.5$ after 40 pulses and then saturates at this level. Simultaneously, the decay time associated with one quasiparticle drops from $\tilde{T}_{1qp} = 20 \mu\text{s}$ to $\tilde{T}_{1qp} \sim 7 \mu\text{s}$. The reduction of $\tilde{T}_{1qp}$ is somewhat surprising, as one might generally expect the decay time per quasiparticle to remain constant as the quasiparticles are pumped away. However, as the number of π pulses increases, the quasiparticles remaining near the junctions generally have higher energy and, hence, cause qubit excitation as well as qubit relaxation. Because $1/\tilde{T}_{1qp}$ is the sum of decay and excitation rates, this conceptually explains, at least in part, the suppression of $\tilde{T}_{1qp}$. Note that despite the introduction of an excitation rate, the qubit will still eventually decay to $|0\rangle$ due to non-quasiparticle relaxation channels (T_{1R}), preventing us from determining the excitation and decay rate separately from the steady-state qubit population. Additionally, the range of values of $\tilde{T}_{1qp}$ reported here is consistent with previous measurements in flux qubits (37).

To further validate the quasiparticle pumping model and rule out alternate explanations of the data, we have also implemented the same pumping scheme, using pulses corresponding to 2π instead of π rotations. If the qubit's environment were directly influenced by the microwave pulses through a mechanism other than quasiparticle pumping (e.g., heating, or saturation of two-level systems), we would expect both the π and 2π pulses to affect the qubit decay time. However, in the experiment we only observe an improvement in the qubit decay when driving the system with π pulses, consistent with the quasiparticle pumping model [(29), section S7].

Having demonstrated that the pumping sequence can substantially reduce the quasiparticle population, we introduced a variable delay before the final probe pulse to investigate how long the reduction in $\langle n_{qp} \rangle$ persists before it returns to the equilibrium value [see (29), section S5]. With the exception of an initial, faster rate for $t_{\text{delay}} < 50 \mu\text{s}$, the return to its steady state is well described by an exponential function with a time constant of $300 \mu\text{s}$. The time scale for quasiparticle recovery is much longer than the qubit lifetime, thus justifying the use of the steady-state solution in Eq. 1 for estimating the quasiparticle population.

The measured quasiparticle population range of $\langle n_{qp} \rangle \sim 0.5$ to 2.5 corresponds to an upper bound on the normalized quasiparticle density of $\chi_{qp} \sim 10^{-4}$ to 10^{-5} , where χ_{qp} is the number of quasiparticles divided by the number of Cooper pairs, and we assume that all decay-inducing quasiparticles are confined to the qubit islands. This is higher than the typical values of $\chi_{qp} \sim 10^{-6}$ to 10^{-7} reported in the literature (7–10). The difference may possibly be attributed to the switching current readout of device A, where the qubit state is inferred by applying a short current pulse to the SQUID and determining its probability to switch to the normal state. Whenever



allow direct comparison. Solid lines are fits to Eq. 1. Each data point is averaged over 10^5 trials. (C) Average quasiparticle number $\langle n_{qp} \rangle$ and (D) decay rate per quasiparticle $\tilde{T}_{1qp}$, extracted from the fits shown in (B). T_{1R} is held constant at $55 \mu\text{s}$ for all fits.

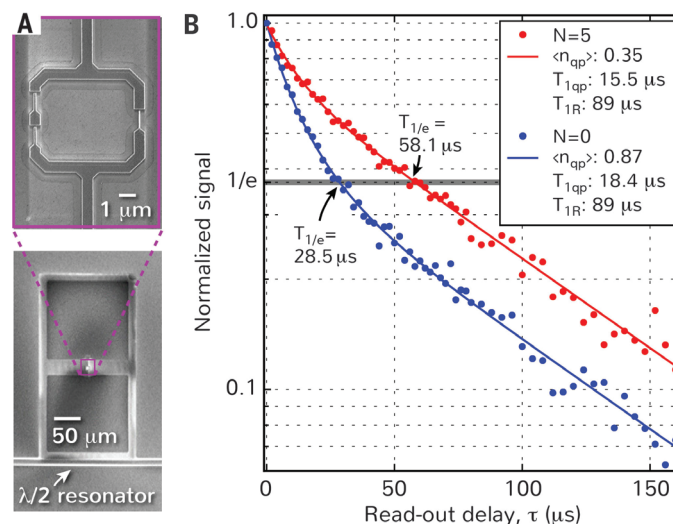


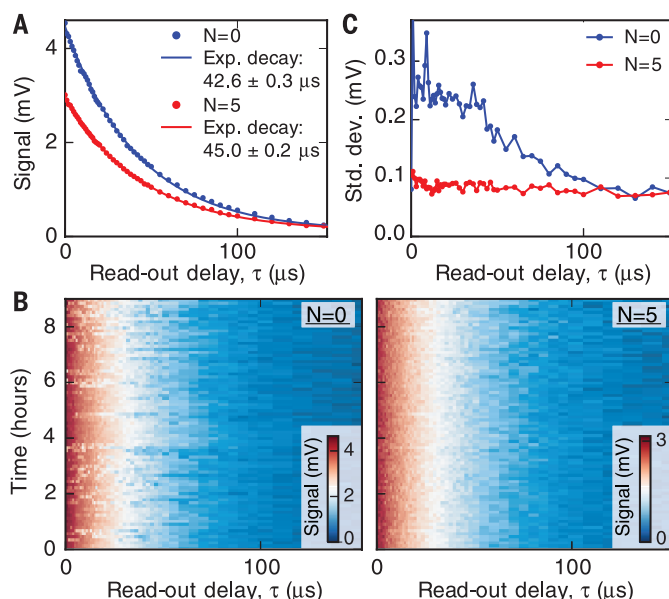
Fig. 3. Improvement in qubit decay time for a C-shunt flux qubit. (A) Scanning electron microscopy image of device B, showing the large square capacitor plates (bottom) and a magnification of the qubit loop containing the three Josephson junctions (top). The qubit is coupled to a half-wavelength ($\lambda/2$) resonator. (B) Qubit relaxation, measured with and without quasiparticle pumping pulses. The trace with $N = 5$ pumping pulses was

taken with a pulse period of $\Delta T = 30 \mu\text{s}$. The data are averaged from 15 individual traces, acquired over a 1-hour period. The fit was performed assuming the same value of T_{1R} for both traces. The uncertainties in fitting parameters are $\langle n_{qp} \rangle \pm 0.02$, $\tilde{T}_{1qp} \pm 3.5 \mu\text{s}$, and $T_{1R} \pm 4 \mu\text{s}$.

Fig. 4. Reduction of qubit coherence variations with quasiparticle pumping. (A)

Averaged qubit decay, measured with and without pumping pulses. The decay function is close to exponential in both cases. The decay time increases by ~6% with the pumping pulses. The traces have not been normalized to account for the decay during the pulse sequence, causing reduced contrast for the data with $N = 5$. The data were measured with $\Delta T = 30 \mu\text{s}$.

(B) Individual traces of the averaged decay data shown in (A), measured without (left) and with five pumping pulses (right). The pumping sequence substantially reduces the temporal fluctuations observed in the decay without pumping pulses. (C) Standard deviation of the data in (B), demonstrating the strong reduction in temporal shot-to-shot fluctuations in the presence of the pumping pulses.



a switching event occurs, quasiparticles are created in close vicinity to the SQUID junctions, leading to an increase in the overall quasiparticle density.

We next investigate quasiparticle pumping in a dispersively read-out C-shunt flux qubit (device B), consisting of a flux qubit loop shunted by a large capacitance (Fig. 3A). Although the capacitor improves the qubit coherence by reducing its sensitivity to charge noise, the C-shunt flux qubit is still affected by quasiparticle fluctuations. As reported in (24), the qubit was observed to switch between a stable configuration, with a purely exponential decay with $T_1 > 50 \mu\text{s}$, and an unstable configuration, with nonexponential decay and temporal fluctuations. The switching between the various configurations was found to occur on a slow time scale, ranging from hours to several days. Similar switching events between stable and unstable configurations have also been observed in a fluxonium qubit and were attributed to fluctuations in the quasiparticle density (22).

We next investigated how the quasiparticle pumping sequence affects the coherence of device B, both in stable and unstable configurations. Because the switching between different configurations is random but slow, we were careful to average only over intervals when no switching event occurred. Figure 3B shows the decay of device B, measured with and without $N = 5$ quasiparticle pumping pulses. The data were taken when the device was in a configuration where the qubit decay was clearly nonexponential, which is well captured by fits to Eq. 1 (solid lines in Fig. 3B). We observed a drop in the quasiparticle population from 0.87 to 0.35, leading to a twofold enhancement in the qubit

decay time. Note that the long-time decay rate is identical for both traces, as expected because the pumping scheme does not affect nonquasiparticle relaxation channels. The results demonstrate that the pumping scheme works even though device B does not have a ground electrode for trapping quasiparticles, but it has been shown that vortices in the capacitor pads can also act as quasiparticle traps (26). The pumping scheme should also be applicable to other qubit modalities in which quasiparticle tunneling contributes to qubit relaxation.

The data in Fig. 3B were acquired by continuously measuring qubit decay traces over a 1-hour period and averaging them together. Figure 4 shows similar repeated measurements of the qubit decay with and without pumping pulses, but these traces were acquired about a week after the data in Fig. 3. In the more recent data set, the qubit is in a configuration where the averaged decay function is relatively well described by a single exponential, both with and without pumping pulses (Fig. 4A), and the five pumping pulses improve the decay time by only ~6%. However, when investigating the individual decay traces (Fig. 4B), we found substantial amounts of noise and temporal fluctuations in the readout signal for the data without pumping pulses. These random variations vanish when implementing the pumping sequence (right panel of Fig. 4B).

To quantify the improvements in variability, we calculated the standard deviation of the readout signal over 9 hours of data (Fig. 4C). With pumping pulses, the standard deviation is independent of the readout delay τ and can be ascribed to the noise of the high-electron-mobility

(HEMT) amplifier used for amplification. Without pumping pulses, the standard deviation is substantially larger for $\tau < 50 \mu\text{s}$ but approaches the same level as for $N = 5$ for long delay times. The increased noise is caused by variations in the qubit T_1 time, which lead to strong fluctuations in the qubit population directly after the initial π pulse. The fluctuations are reduced as the qubit decays to the ground states for long τ , leaving only the contributions from the HEMT noise.

Our implementation of a stochastic scheme to dynamically shape the environment by pumping quasiparticles in a superconducting flux qubit lead to substantial improvements in both qubit coherence times and coherence variability. In addition to applications in superconducting qubits, we anticipate our results to be of practical importance for implementing Majorana fermions in hybrid semiconductor-superconductor systems, where the presence of a single quasiparticle is detrimental to the device performance (25).

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Supplementary Text

Figs. S1 to S4
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QUANTUM OPTICS

Quantum optical circulator controlled by a single chirally coupled atom

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Integrated nonreciprocal optical components, which have an inherent asymmetry between their forward and backward propagation direction, are key for routing signals in photonic circuits. Here, we demonstrate a fiber-integrated quantum optical circulator operated by a single atom. Its nonreciprocal behavior arises from the chiral interaction between the atom and the transversally confined light. We demonstrate that the internal quantum state of the atom controls the operation direction of the circulator and that it features a strongly nonlinear response at the single-photon level. This enables, for example, photon number-dependent routing and novel quantum simulation protocols. Furthermore, such a circulator can in principle be prepared in a coherent superposition of its operational states and may become a key element for quantum information processing in scalable integrated optical circuits.

As their electronic counterparts, integrated optical circuits require nonreciprocal elements, such as diodes and circulators, for signal routing and processing. Bulk optical implementations of such components are readily available and rely mostly on a nonreciprocal polarization rotation via the Faraday effect. However, this mechanism cannot straightforwardly be translated to integrated optics because nano-optical structures are typically birefringent (*1*). Demonstrations of integrated nonreciprocal devices therefore rather used, for example, nonlinear optical effects (*2–4*), time-modulation of the waveguide (*5–7*), or magneto-optical effects in conjunction with the extraordinary polarization properties of strongly confined light fields (*1, 8, 9*). None of these approaches could simultaneously realize strong nonreciprocity, low loss, and compatibility with low light levels. However, these characteristics are crucial when it comes to quantum applications such as quantum communication (*10*), quantum information processing (*11*), and quantum simulation (*12*). There, information is encoded in individual photons, and their loss must be avoided as much as possible. This condition narrows down the scope of quantum-compatible nonreciprocal optical elements to nonreciprocal phase shifters and circulators.

We experimentally realized a fiber-integrated circulator that is capable of routing individual

photons for quantum optical applications. It is operated by a single atom that is coupled to the evanescent field of a whispering-gallery-mode (WGM) microresonator. The latter is interfaced with two coupling fibers (*13, 14*), realizing a four-port device (Fig. 1A). We demonstrate that the internal quantum state of the atom controls the operation direction of the circulator. Furthermore, we show that being controlled by a single atom, our device features photon number-dependent routing capability that has application in, for example, novel quantum simulation protocols.

In order to achieve efficient routing, the coupling rates κ_a and κ_b between the resonator field and the field in the respective coupling fiber “a” or “b” are adjusted so that both fibers are approximately critically coupled to the empty resonator: $\kappa_a \approx \kappa_b \gg \kappa_0$, where κ_0 is the intrinsic resonator field decay rate. When no atom is coupled to the resonator mode, this realizes an add-drop filter (*13, 14*) in which light that is launched into one fiber will be transferred to the other fiber via the resonator. Because of its strong transverse confinement, the evanescent field of the clockwise (cw) propagating resonator mode is almost fully circularly polarized (*15*). Its electric field vector rotates counterclockwise in the plane orthogonal to the resonator axis (z axis), corresponding to σ^- polarization (Fig. 1B). Time-reversal symmetry then implies that the evanescent field of the counterclockwise (ccw) propagating mode is almost fully σ^+ -polarized (*15*). Coupling such “spin orbit-locked” light fields to single quantum emitters gives rise to the new paradigm of chiral quantum optics (*16*). In the context of

WGMs, it recently enabled the implementation of an optical switch controlled by a single photon (*17*) and the extraction of a single photon from an optical pulse (*18*). Moreover, an optical isolator was realized that either transmits or dissipates fiber-guided light depending on its propagation direction (*19*). For the circulator, we resonantly coupled a single ^{85}Rb atom to the resonator, which is prepared in the outermost Zeeman sublevel $m_F = +3$ of the $5S_{1/2}$, $F = 3$ hyperfine ground state. Thus, the two counter-propagating resonator modes couple to an effective V-level system (Fig. 1C). Remarkably, the strength of the transition to the $5P_{3/2}$, $F' = 4$, $m_{F'} = +4$ excited state is 28 times stronger than that to the $F' = 4$, $m_{F'} = +2$ state (*20*). As a consequence, light in the ccw mode interacts strongly with the atom with a coupling strength g_{ccw} , whereas light in the cw mode exhibits much weaker coupling, $g_{\text{cw}} \ll g_{\text{ccw}}$. This chiral (direction-dependent) light-matter interaction breaks Lorentz reciprocity (*19, 21–25*). In particular, the presence of the atom changes the resonator field decay rate from $\kappa_{\text{tot}} = \kappa_0 + \kappa_a + \kappa_b$ to $\kappa_{\text{tot}} + \Gamma_{\text{cw/ccw}}$, where $\Gamma_{\text{cw/ccw}} = g_{\text{cw/ccw}}^2/\gamma$ is the direction-dependent atom-induced loss rate (*26*) and $\gamma = 2\pi \times 3$ MHz is the dipole decay rate of Rb. For light in the cw mode, Γ_{cw} is small, and the field decay rate is not substantially modified by the atom, whereas for the ccw mode, Γ_{ccw} can become comparable with or even exceed κ_{tot} . Consequently, the add-drop functionality is maintained when light is launched into those fiber ports for which it couples to the cw mode (Fig. 1D, input ports 2 and 4). However, for the two other input ports (Fig. 1D, 1 and 3), the light couples to the ccw mode, and the resonator-atom system operates in the undercoupled regime

$$\kappa_a, \kappa_b \ll \Gamma_{\text{ccw}} \quad (1)$$

Thus, the incident light field remains in its initial fiber. Overall, the device thus realizes an optical circulator that routes light from the input port i to the adjacent output port $i + 1$ with $i \in \{1, 2, 3, 4\}$ (Fig. 1D). Preparing the atom in the opposite Zeeman ground state, $F = 3$, $m_F = -3$, exchanges the roles of the cw and ccw mode and thus yields a circulator with reversed operation direction. Hence, the circulator is programmable, and its operation direction is defined by the internal state of the atom.

For nonperfect circular polarization of the modes and our experimental parameters ($g_{\text{ccw}} \approx 2\pi \times 12$ MHz), the ratio between $\Gamma_{\text{ccw}} \approx 2\pi \times 48$ MHz and $\Gamma_{\text{cw}} \approx 2\pi \times 1.7$ MHz is finite (*26*). Concerning the performance of the circulator, there is thus a trade-off between efficient light transfer from

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one fiber to the other via the cw mode, which implies $\kappa_a, \kappa_b \gg \kappa_0 + \Gamma_{\text{cw}}$ and the condition that the presence of the atom should substantially influence the field decay rate (Eq. 1). To find the optimum working point in our experiment, we measured the circulator performance as a function of the fiber-resonator coupling strengths

κ_a and κ_b , which can be adjusted by changing the distance between the respective fiber and the resonator surface. We imposed the constraint that fiber a is critically coupled to the empty resonator that is loaded with fiber b: $\kappa_a = \kappa_b + \kappa_0$ (26). For each setting, we measured the transmissions T_{ij} to all output ports j when sending

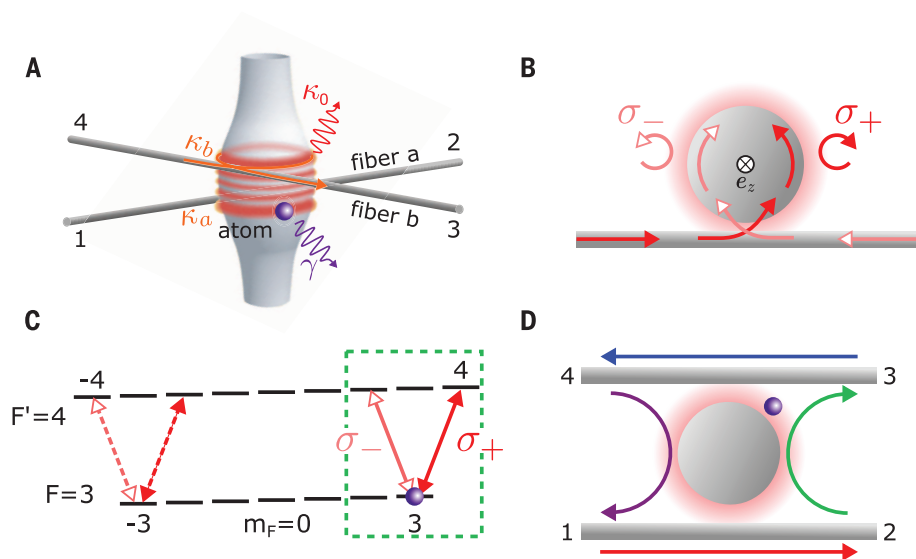


Fig. 1. Operation principle of the circulator. (A) Schematic of the experimental system. A single ^{85}Rb atom is coupled to the WGM of a bottle microresonator, which is interfaced by two tapered fiber couplers. (B) The polarization of the evanescent field of the modes, σ^- and σ^+ , depends on their propagation direction, clockwise or counterclockwise. (C) For a ^{85}Rb atom prepared in the $F = 3$, $m_F = +3$ Zeeman state, the transition strength for σ^+ -polarized light (for the counterclockwise mode) is 28 times larger than for σ^- -polarized light (for the clockwise mode). This situation is reversed if the atom is prepared in $m_F = -3$. (D) Routing behavior of the circulator for the atom prepared in $m_F = +3$.

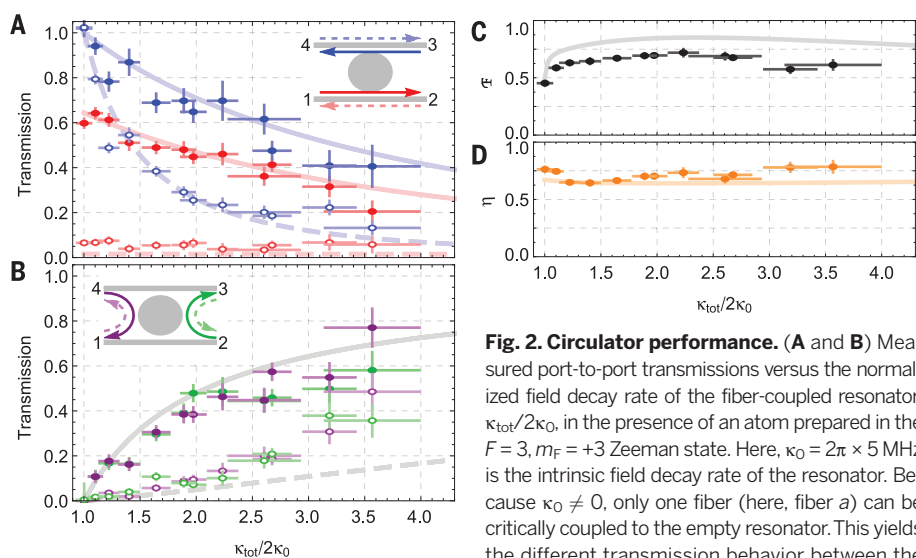


Fig. 2. Circulator performance. (A and B) Measured port-to-port transmissions versus the normalized field decay rate of the fiber-coupled resonator, $\kappa_{\text{tot}}/2\kappa_0$, in the presence of an atom prepared in the $F = 3$, $m_F = +3$ Zeeman state. Here, $\kappa_0 = 2\pi \times 5$ MHz is the intrinsic field decay rate of the resonator. Because $\kappa_0 \neq 0$, only one fiber (here, fiber a) can be critically coupled to the empty resonator. This yields the different transmission behavior between the

cases $1 \leftrightarrow 2$ and $3 \leftrightarrow 4$. The solid circles indicate transmissions to the forward direction, and the open circles indicate transmissions to the backward direction; the color code follows that of the insets. (C and D) Operation fidelity $\mathcal{F}$ and photon survival probability η of the circulator, calculated from the data in (A) and (B). The lines are the predictions of our theoretical model (26), with the atom-resonator coupling strength $g_{\text{ccw}} = 2\pi \times 12$ MHz. The vertical error bars indicate $\pm 1\sigma$ statistical errors, and the horizontal error bars represent an estimate of the variation of κ_{tot} due to drifts of the fiber-resonator distances during the corresponding measurement.

a weak coherent probe field into the four different input ports i (26). The relevant transmissions as a function of κ_{tot} are shown in Fig. 2, A and B.

To quantify the performance of the circulator, we calculated the overlap of the renormalized transmission matrix $\tilde{T} = (T_{ij}/\eta_i)$, with the one expected for the ideal circulator, T^{id} (Fig. 3A). Here, $\eta_i = \sum_k T_{ik}$ is the survival probability of a photon entering port i . Thus, the average operation fidelity of our circulator is

$$\mathcal{F} = \frac{\text{Tr}[\tilde{T} \cdot T^{\text{id}T}]}{\text{Tr}[T^{\text{id}} \cdot T^{\text{id}T}]} = 1 - \frac{1}{8} \sum_{i,j} |\tilde{T}_{i,j} - T^{\text{id}}_{i,j}| \quad (2)$$

This quantity—also called inquisition (27)—gives the probability of a correct circulator operation averaged over its eigenstates. The minimum fidelity is $\mathcal{F} = 0$, whereas $\mathcal{F} = 1$ is reached for ideal operation. For any reciprocal device ($\tilde{T} = \tilde{T}^T$), the fidelity is bound by $\mathcal{F} \leq 0.5$. In Fig. 2, C and D, we plot $\mathcal{F}$ and the average photon survival probability $\eta = \sum_i \eta_i/4$ as a function of κ_{tot} . The results show an optimum circulator performance for $\kappa_{\text{tot}}/2\kappa_0 = 2.2$, where $\mathcal{F} = 0.72 \pm 0.03$ and, at the same time, $\eta = 0.73 \pm 0.04$. The measured transmission matrix (T_{ij}) for the optimum working point (Fig. 3B) shows good qualitative agreement with T^{id} . In order to demonstrate that the chiral atom-light coupling is at the origin of the nonreciprocal behavior, we also measured the transmission matrix without coupled atom and obtained a symmetric matrix (Fig. 3D). The circulator performance can also be quantified by the isolations, $I_i = 10 \log(T_{ii+1}/T_{i+1,i})$, of the four optical diodes formed between adjacent ports. For the optimum working point, we obtained $(I_i) = (10.9 \pm 2.5, 6.8 \pm 1.3, 4.7 \pm 0.7, 5.4 \pm 1.1)$ dB and an average insertion loss of $-10 \log \eta = 1.4$ dB.

In order to reverse the operation direction of the circulator, we then prepared the atom in the opposite Zeeman ground state, $F = 3$, $m_F = -3$ (26). This results in a complementary V-type level scheme (Fig. 1C, dashed arrows). For this case, we obtained the transmission matrix shown in Fig. 3C, again measured for the optimum fiber-resonator coupling rate. Here, we observed a fidelity with respect to the reversed circulator of $\mathcal{F} = 0.70 \pm 0.02$, an average photon survival probability of $\eta = 0.69 \pm 0.02$ corresponding to 1.6 dB average insertion loss, and optical isolations of $(I_i) = -(8.3 \pm 0.8, 4.9 \pm 0.7, 3.7 \pm 0.4, 5.6 \pm 0.5)$ dB. Taking into account the sign change, these results agree well with the values obtained for the atom in the $F = 3$, $m_F = +3$ state. State-of-the-art passive integrated optical circulators exhibit comparable isolations of ~ 12 dB, but they are subject to orders-of-magnitude-higher insertion losses of ~ 30 dB (8, 9).

Last, in the regime of strong coupling, a single photon already saturates the atom (14, 17, 28). Thus, the transmission properties for the case of two photons simultaneously impinging on the circulator should strongly differ from the single-photon case. In order to demonstrate this quantum nonlinearity, we measured second-order

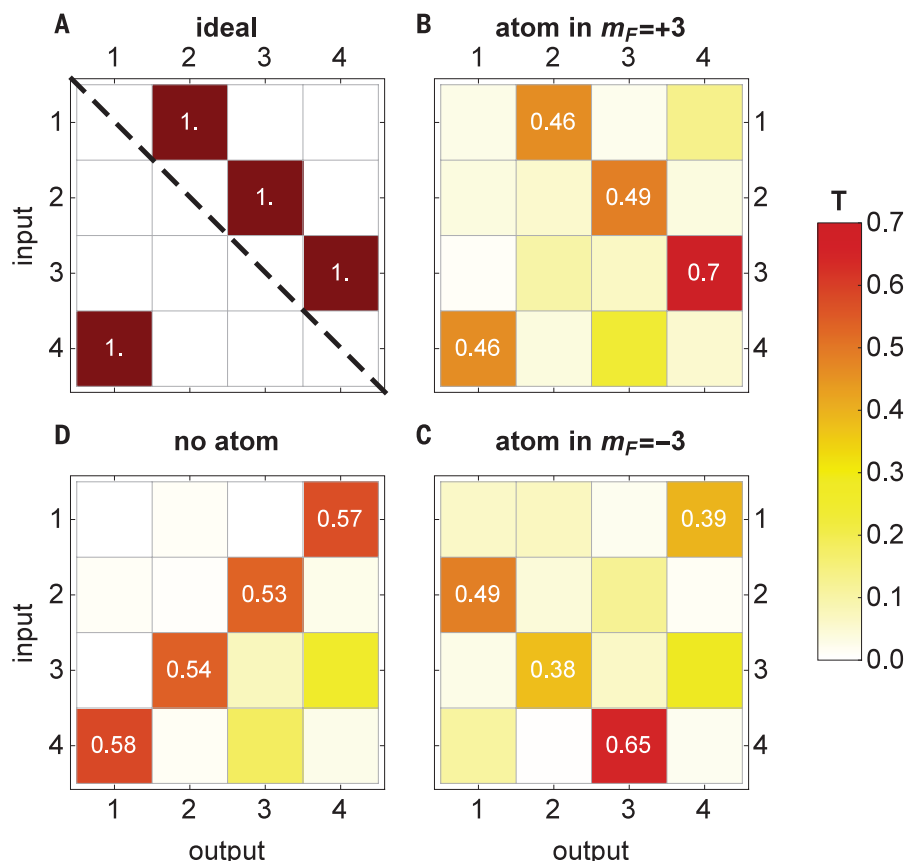


Fig. 3. Transmission matrices (T_{ij}). The rows correspond to the input ports i and the columns to the output ports j . (A) Transmission matrix for an ideal circulator. The broken symmetry with respect to the dashed line indicates the nonreciprocal character of the device. Transmission matrix measured for the atom prepared in (B) $F = 3$, $m_F = +3$ and (C) $F = 3$, $m_F = -3$ (reversed operation direction). For comparison, (D) shows the symmetric transmission matrix of the system measured without atom. For (B), (C), and (D), the fiber-resonator coupling was set to the optimal working point $\kappa_{\text{tot}}/2\kappa_0 = 2.2$. In (A) to (D), the four highest transmission values are given. All other values are below 0.28 (table S1).

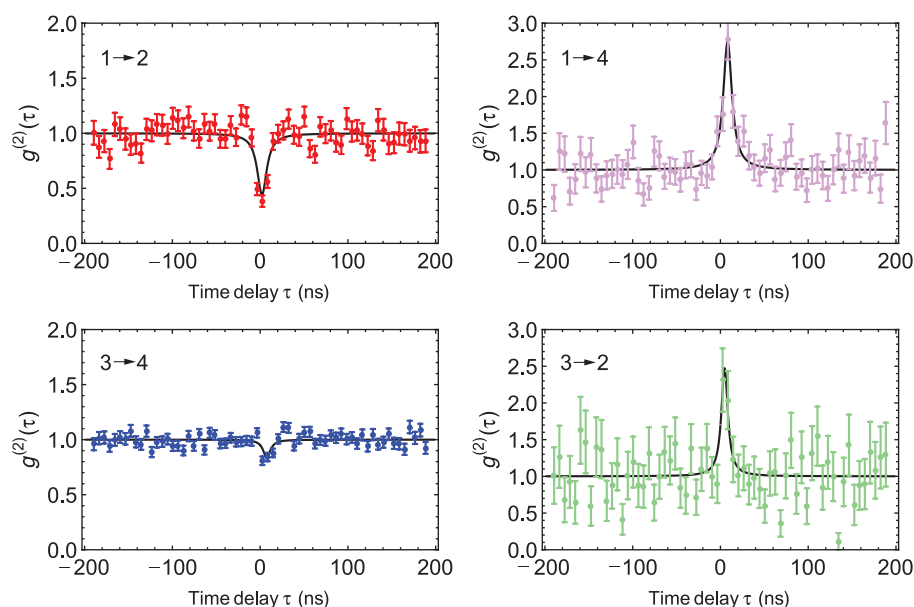


Fig. 4. Quantum nonlinearity of the circulator.

Second-order correlation, $g^{(2)}(\tau)$, as a function of the detection time delay τ between pairs of photons, normalized so that $g^{(2)}(\tau) = 1$ for $\tau \gg 1/\kappa_{\text{tot}}$. The labels $i \rightarrow j$ indicate the input and output ports for the respective measurements. We use the same color code as in Fig. 2; the solid lines are guides to the eye. The error bars indicate the $\pm 1\sigma$ statistical error.

correlation functions for all input-output combinations when the atom is prepared in $F = 3$, $m_F = +3$ (fig. S2). Those second-order correlations that yield the strongest signals are shown in Fig. 4. As expected (26), the strongest signals occur for the cases in which the photons couple into the ccw resonator mode. We observed photon

antibunching for photons that remain in the initial fiber (forward direction of the circulator, $1 \rightarrow 2$, $3 \rightarrow 4$) and bunching for photons that are transmitted to the other fiber (backward direction of the circulator, $1 \rightarrow 4$, $3 \rightarrow 2$). This behavior illustrates the photon number-dependent routing capability; while individually arriving

photons remain in their original fiber, simultaneously arriving photons are preferentially transferred to the other fiber.

The demonstrated circulator concept is useful for the processing and routing of classical signals at ultralow light levels in integrated optical circuits and networks. Beyond that, and in contrast to

dissipative nonreciprocal devices, a circulator that is controlled by a single quantum system also enables operation in coherent superposition states of routing light in one and the other direction, providing a route toward its application in future photonic quantum protocols. The demonstrated operation principle is universal in the sense that it can straightforwardly be implemented with a large variety of different quantum emitters provided that they exhibit circularly polarized optical transitions and that they can be spin-polarized. Using state-of-the-art WGM microresonators (29), one could realize a circulator with optical losses below 7% and close-to-unit operation fidelity (26). This would then allow one to almost deterministically process and control photons in an integrated optical environment. Arranging N circulators so that they form a linear array allows one to realize a $(2N + 2)$ -port optical circulator. Moreover, two- and three-dimensional networks of quantum circulators are potential candidates for implementing lattice-based quantum computation (30). Such networks would enable the implementation of artificial gauge fields for photons (31–33), in which a nonlinearity at the level of single quanta allows for the flux to become a dynamical degree of freedom that interacts with the particles themselves (34).

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NANOMATERIALS

Emergence of hierarchical structural complexities in nanoparticles and their assembly

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We demonstrate that nanoparticle self-assembly can reach the same level of hierarchy, complexity, and accuracy as biomolecules. The precise assembly structures of gold nanoparticles (246 gold core atoms with 80 *p*-methylbenzenethiolate surface ligands) at the atomic, molecular, and nanoscale levels were determined from x-ray diffraction studies. We identified the driving forces and rules that guide the multiscale assembly behavior. The protecting ligands self-organize into rotational and parallel patterns on the nanoparticle surface via C-H... π interaction, and the symmetry and density of surface patterns dictate directional packing of nanoparticles into crystals with orientational, rotational, and translational orders. Through hierarchical interactions and symmetry matching, the simple building blocks evolve into complex structures, representing an emergent phenomenon in the nanoparticle system.

Hierarchical self-assembly of nanoparticles (NPs) into complex architectures across different length scales is an important capability in nanotechnology (1–4), especially for the bottom-up fabrication for electronics, sensors, energy conversion, and storage devices. Such self-assembly can be driven by entropy-dictated maximization of the packing density, as demonstrated in close packing of spheres, binary NPs, rods, and hard polyhedrons (5–9). Interparticle interactions, such as the electrostatic attraction (10), cDNA binding (11, 12), and patchy NP surfaces (13–15), have also been exploited to guide assembly into diverse lattice structures. Despite these advances, NP assembly has not achieved the same level of atomic accuracy as in biological systems.

We now demonstrate that NP-assembled structures can reach the same hierarchy and atomic accuracy as biomolecules at the interparticle and intraparticle levels. Through crystallization of uniform 2.2-nm gold NPs bearing *p*-methylbenzenethiolate (*p*-MBT) surface ligands [Au₂₄₆(*p*-MBT)₈₀], we fully resolve the entire self-

assembled structures at atomic (packing of gold atoms), molecular (packing of surface ligands), and nanoscale (packing of NPs) levels by single-crystal x-ray diffraction (SC-XRD) (16). The precise structural information across scales allows an in-depth examination of the forces and the rules that govern the assembly behavior at each level. We reveal that the simple structure of protecting ligands can generate complex patterns on the NP surfaces, and the symmetry and density of the surface patterns further guide the packing of NPs into lattices with orientational, rotational, and translational order.

The Au₂₄₆(*p*-MBT)₈₀ NPs were synthesized by a two-step “size-focusing” method (16). Briefly, the ligand-coated NPs in a narrow size range from ~10 to ~70 kilodaltons were first made, and then the size focusing process gradually led to the stable Au₂₄₆(*p*-MBT)₈₀ NPs (figs. S1 and S2). The as-obtained product (~90% purity) was subject to further solvent fractionation to reach molecularly pure Au₂₄₆ NPs. Optical absorbance spectra showed a prominent peak at 470 nm and several weak humps at 400 and 600 nm (fig. S3), indicating the nonplasmonic nature of the Au₂₄₆(*p*-MBT)₈₀ NPs. Single crystals were grown by diffusion of antisolvent (acetonitrile) into a toluene solution of the pure Au₂₄₆ NPs. The structure was determined at the resolution of 0.96 Å by SC-XRD

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(tables S1 and S2). The individual NPs have a nearly spherical shape, with a metal core diameter of 2.2 nm and an overall diameter (including the ligand shell) of 3.3 nm (fig. S4).

In contrast to conventional spherical NPs, which are typically packed into superlattices with simple translational symmetry, such as face-centered cubic (fcc) or body-centered cubic (bcc) (11, 17), the $\text{Au}_{246}(\text{p-MBT})_{80}$ NPs packed into a more complex monoclinic lattice (Fig. 1). In the (001) plane of the crystal lattice, the NPs are organized into a square lattice (Fig. 1A), akin to the packing mode in the {100} plane of the fcc lattice. The interparticle distance of 3.1 nm (less than the 3.3 nm overall size of the NP) arose from ligand interlocking. These two-dimensional (2D) lattices stacked up obliquely, instead of perpendicularly along the z direction (Fig. 1B), so the overall 3D lattice deviated from the fcc lattice. The NP packing density of ~60% is less than the 74% of the fcc lattice. Such monoclinic packing should be driven by specific interparticle interactions, because entropy alone would favor close packing (5–9). Indeed, when zooming into the surfaces of the NPs, the monoclinic packing was correlated to the alignment of surface ligands among NPs (Fig. 1, D to F, green, blue, red).

The p -MBT ligands were highly ordered and self-organized into two different patterns on the

gold sphere (Fig. 2A). At the pole site of the gold sphere, 25 of the p -MBTs were rotationally arranged into four pentagonal circles (Fig. 2B, highlighted in red, blue, blue, green). Each circle had the same “latitude” and rotational direction. Instead of “on-top” adsorption, the thiolates (characterized by S-C bond vectors) “tilted” away from the radial direction of the gold sphere because of the gold-thiolate binding geometry (18). This pattern we call α -rotation created a “singularity” (19) at the pole (Fig. 2B). At the waist site, six of the p -MBT ligands were aligned into three alternating parallel pairs to form a pattern we call β -parallel (Fig. 2C). Five of these β -parallel patches circled up and covered the waist of the NP. The packing densities of ligands are ~14 ligands nm^{-2} for α -rotation and ~6 ligands nm^{-2} for β -parallel as measured based on the surface area of the inner gold sphere (Fig. 2A, magenta polyhedron). The clockwise and counterclockwise rotational arrangement of p -MBT ligands induces chirality in the NP, and both chiral isomers (denoted R/L) participate in the crystal packing (Fig. 1, C and F). The NPs with the same chirality are packed in the same square layer, and the neighboring square layers are composed of NPs with opposite chirality (Fig. 1C).

Such rotational and parallel self-assembled surface patterns of ligands are reminiscent of

the α helix and β sheet in proteins, which suggests that NPs could exhibit a level of structural complexity comparable to that of biomolecules. The secondary structures of proteins are mainly stabilized by the hydrogen bonds. Here, the surface patterns on the NPs are stabilized by intermolecular C-H $\cdots\pi$ interactions, in which the C-H bonds from the phenyl rings or the methyl groups interact with the π electrons (Fig. 2D). Such intermolecular interactions were observed in the packing structures of aromatic molecules and supramolecules, and the strength is about 1.5 to 2.5 kcal mol^{-1} (20, 21). Specifically, the C-H $\cdots\pi$ interactions in the α -rotation linked the 25 p -MBT ligands into five spirals, with the H $\cdots\pi$ distances ranging from 2.5 to 3.0 Å and C-H- π angle ranging from 112° to 147° (fig. S5). For the β -parallels, the alternating pattern among the three pairs was also stabilized by the C-H $\cdots\pi$ interactions (fig. S6). We reason that the surface patterns are intrinsic to NPs instead of being induced by crystallization, because the collective C-H $\cdots\pi$ interactions can generate an energy barrier and stabilize the pattern, similar to the case in which the hydrogen bonds can stabilize the DNA double helix in solution.

To study the interparticle interactions, we isolated the coordination environment of NPs

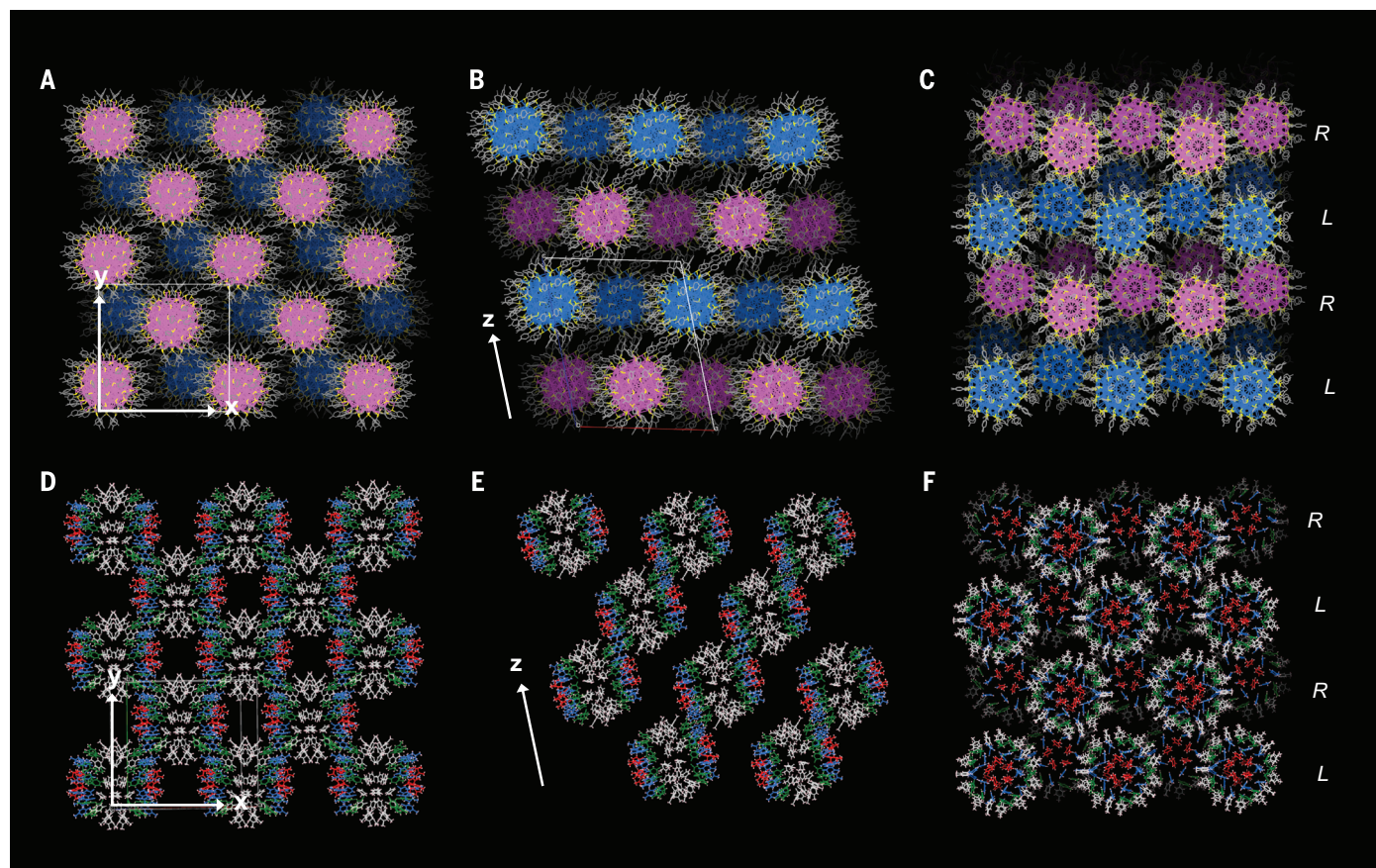


Fig. 1. Packing structure of the derivatized Au NPs in single crystals. (A to C) View from z direction (A), y direction (B), and x direction (C). Magenta, blue: Au NPs with different chirality; yellow: sulfur; gray: carbon. (D to F) Alignment of surface ligands among the NPs. Gray: ligands located at the waist of the NP; red, blue, green: ligands located at the poles.

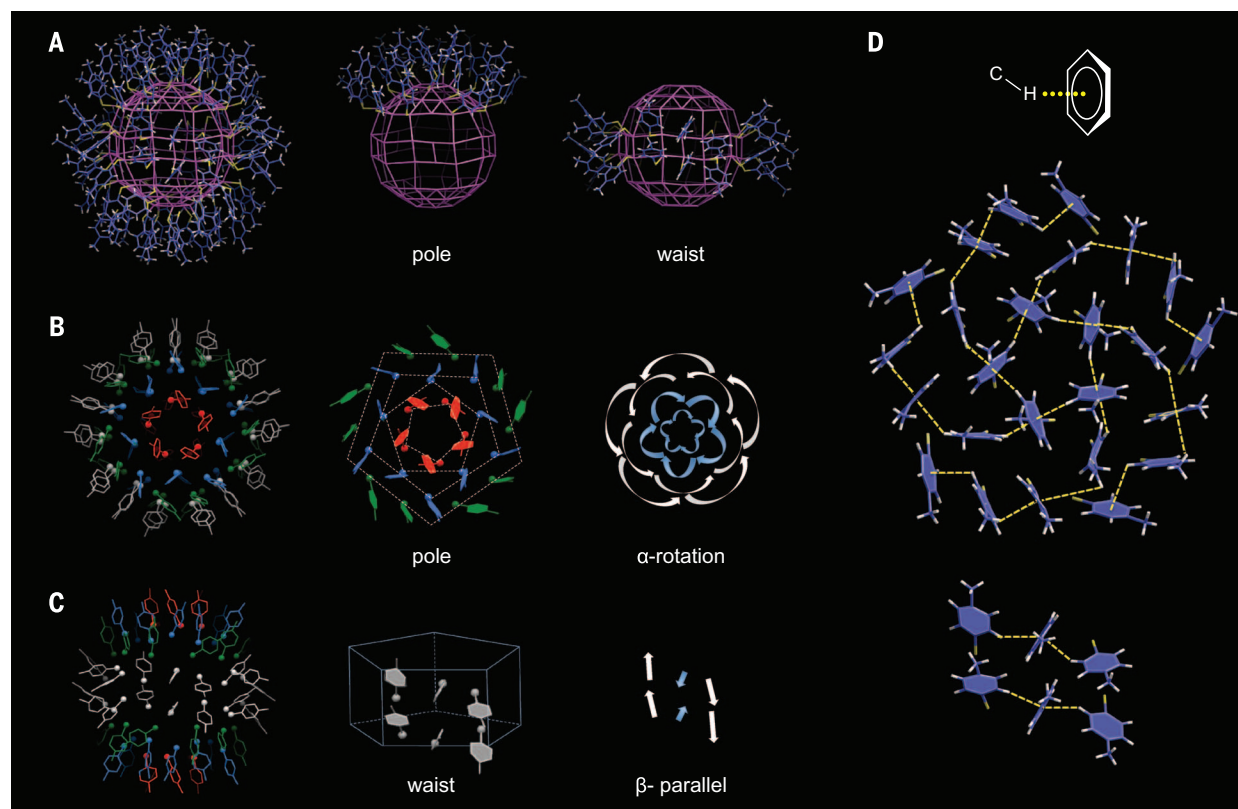


Fig. 2. Self-assembled surface patterns of the ligands on the Au NPs. (A) Overall structure of ligands on the surface of NPs. (B) Rotational packing of ligands at the pole site of the NP. (C) Parallel packing of ligands at the waist of the NP. (D) The C-H... π interactions for stabilizing the large-scale rotational patterns and parallel patterns.

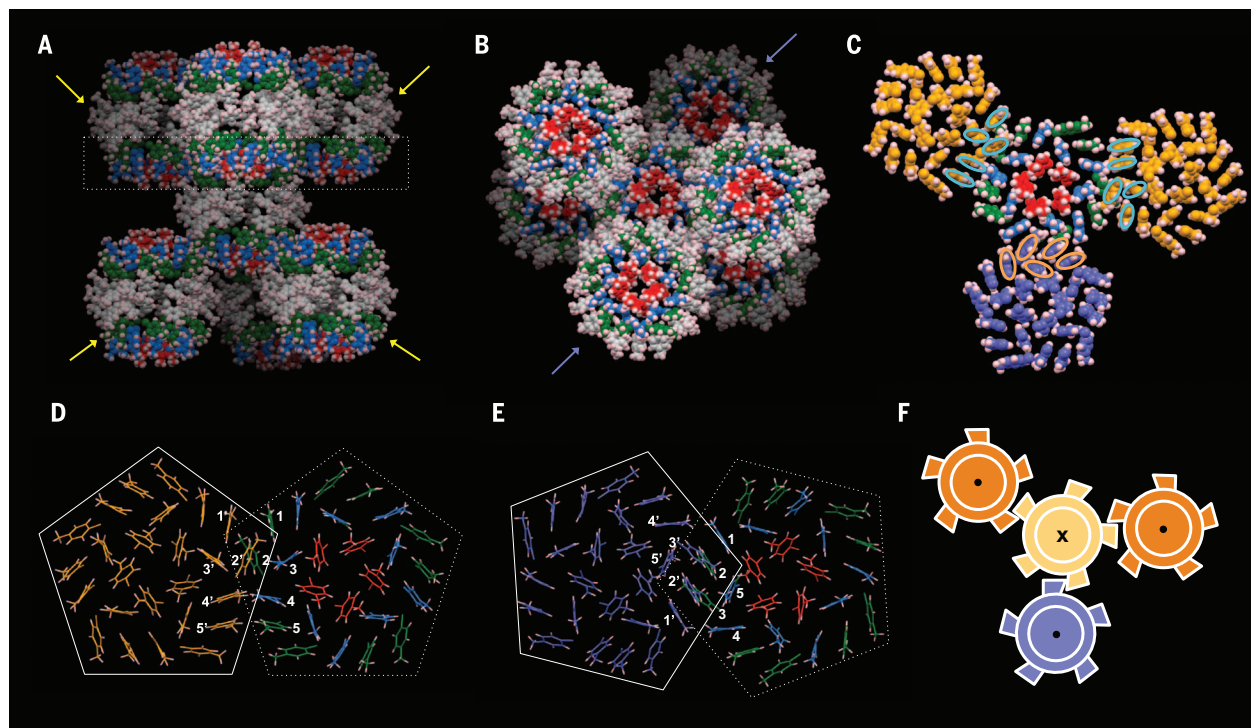


Fig. 3. Interparticle self-assembly dictated by the ligand density and the symmetry of surface patterns. (A and B) Coordination geometry of NPs in the crystal lattice: side view (A) and top view (B). (C) Contacting environment among the interparticle ligands. The outside pentagons are located at the bottom of the top three nanoparticles in (A), and the central pentagon is located at the top of the central nanoparticle. (D) Side-by-side stacking of the ligands in the NPs with the same chirality. (E) Point-to-point stacking of the ligands in the NPs with opposite chirality. (F) Scheme showing the directional packing of NPs achieved through matching the symmetry of surface patterns.

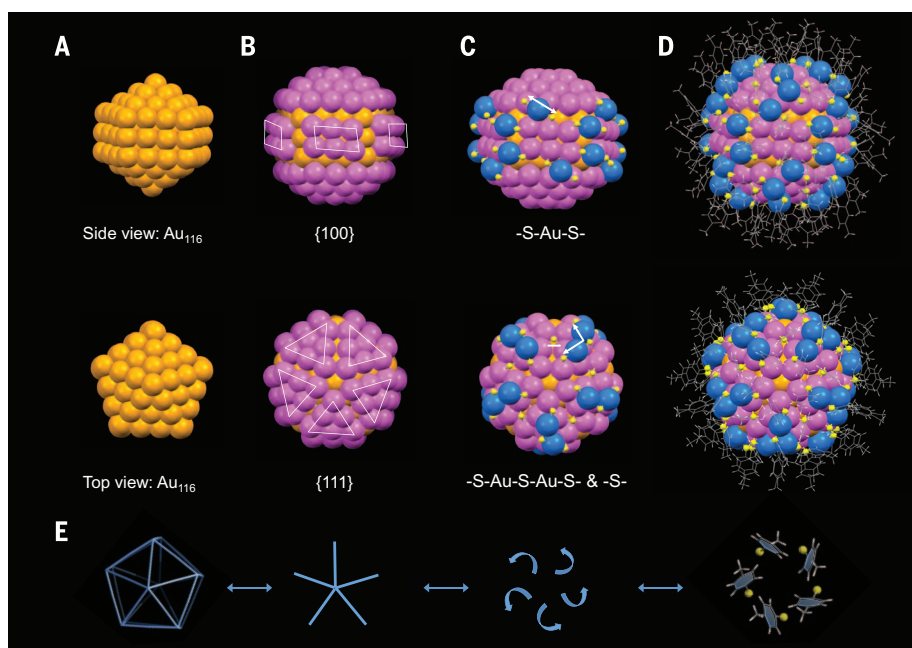


Fig. 4. Intraparticle self-assembly in the Au NP. (A) Au₁₁₆ *i*-Dh kernel, top: side view; bottom: top view. (B) Transition layer structure as colored in magenta. (C) Gold-sulfur interfacial structure containing diverse surface protecting motifs. (D) Surface carbon layer and overall structure. (E) Intraparticle symmetry matching and emergent behavior.

(Fig. 3), which reflects the orientational, rotational, and translational symmetry of the crystal lattice. Each NP has six nearest neighbors. Four of them were within the same square layer and had the same chirality as the central one (Fig. 3A, yellow arrows). The particle-to-particle distance was 31.0 Å, whereas the other two had opposite chirality (Fig. 3B, purple arrows) and the distance was 31.7 Å. These NPs interacted with the central one by aligning the α -rotation ligands at the pole sites, whereas there were fewer interactions of the β -parallel ligands at the waist site. The preferred alignment of α -rotation ligands was correlated to the higher packing density of ligands in α -rotation ($\sim 14 \text{ nm}^{-2}$) compared to the density in β -parallel ($\sim 6 \text{ nm}^{-2}$). In this way, the van der Waals interaction among the contacting ligands was maximized. This surface-density-dictated assembly strategy is in accordance with the assembly behavior observed in DNA-coated NPs (11, 12, 22), oleic acid-protected nanoplates (15), and nanocrystals (17).

In addition to the maximization of ligand interactions, matching of surface symmetry was also important for ordered NP assembly. For a more detailed picture on interparticle interactions, we further isolated the contacting region among the top three and the central NPs (Fig. 3A, white frame), as reflected in the four pentagons shown in Fig. 3C. Notably, the central NP spontaneously matched with the same surface region of its neighbors when forming packing interaction (Fig. 3, C to E). Each contacting area was composed of about five pairs of symmetrically identical *p*-MBT ligands located at the corners of the contacting pentagons (Fig. 3, D and E). For the neighboring NPs with the same chirality as the central one,

the pentagons were aligned side by side (Fig. 3D), whereas for the opposite chirality, the pentagons were aligned point to point (Fig. 3E). The spacing between interacting ligands was $\sim 2.5 \text{ Å}$, and the shortest spacing could reach 1.9 Å (fig. S7). This short distance indicates tight packing among NPs. The symmetry-matching strategy is akin to the assembly of gears (Fig. 3F), with each contacting area resembling a tooth of the gear. Matching the symmetry facilitates the interlocking of surface ligands. Each pentagon has five potential contacting areas to connect with five other NPs, but only three are occupied because of the limited space around the NP.

The assembly structure provides a precise depiction of the long-standing issue of ligand effects on the packing structures of NPs (23, 24). It implies that the inhomogeneous but symmetric distribution of surface ligands can serve as “sticky bonds” for directional NP assembly. Such spontaneously organized surface patterns have an effect similar to that of artificially decorated surface patches in guiding assembly (3, 13, 14). The perfect uniformity of the NPs was critical for maintaining the fidelity of surface patterns. The packing behavior also represents an emergent phenomenon (25, 26), in which small and simple entities (the surface ligands), through multiscale interactions, generate larger and more complex structures with new features that are not manifested in the simple entities. If the *p*-MBT ligand is viewed as the primary structure, then through the C-H $\cdots\pi$ interactions among the ligands, a more complex secondary structure of surface patterns is generated, and these surface patterns, via the density- and symmetry-dictated packing rules, further guide the packing of NPs

into the tertiary structure of the crystal lattice. It is expected that by controlling the symmetries of surface ligand patterns, diverse packing structures of NPs could be achieved.

The assembly behavior within each NP also exhibits such order and hierarchy. The individual NP can be divided into four regions from the core to surface. The innermost part is a three-shell Au₁₁₆ Ino decahedron (*i*-Dh) exposing {111} facets at the poles and {100} facets at the waist (Fig. 4A and fig. S8). The *i*-Dh is one of the energy minima for packing of metal atoms (27–29). The second part is a transition layer containing 90 gold atoms (Fig. 4B, magenta), which “sphericizes” the *i*-Dh, lowers the surface energy, and provides “foot-holds” for anchoring surface protecting motifs. The two parts together give rise to an Au₂₀₆ core. The average Au-Au bond length in the *i*-Dh was $2.87 \pm 0.05 \text{ Å}$ (fig. S9), whereas the bonds associated with the transition layer showed larger deviations ($2.89 \pm 1.12 \text{ Å}$) because of the binding effect of surface ligands. The third part is the Au-S interfacial layer, in which the surface dangling bonds of the Au₂₀₆ core are anchored by the protecting motifs assembled from 40 gold and 80 sulfur atoms. The protecting motifs are highly diverse, in accordance with the rich surface features of the Au₂₀₆ core (such as facets and grooves, Fig. 4B). At the poles, the exposed {111} facets are protected by –S–Au–S–Au–S– motifs and the {111}|{111} grooves are linked by simple bridging thiolates (Fig. 4C, bottom); at the waist, the {100}|{100} and {111}|{100} grooves are all covered by –S–Au–S– staple motifs (Fig. 4C, top). The fourth part is the surface carbon layer as discussed above (Fig. 4D). Although each part of the NP followed different assembling rules, the five-fold symmetry was always maintained (Fig. 4E). An intriguing question is whether the packing symmetry of the surface patterns emerged from the core or the core symmetry emerged from the surface. Although each part of NP contributes to the overall energy minimization, we deduce that the surface ligands play a pivotal role in guiding the intraparticle assembly, because the gold atoms are less selective when packing into specific structure types such as fcc, decahedron, or icosahedron (30). In addition, the rotational patterns of ligands induce the chirality in the interfacial layer and transitional layer (figs. S10 and S11), and packing of gold atoms alone cannot give rise to chirality. Also, the larger sphere of the ligands likely has a greater surface energy to minimize, and the structures of Au NPs were highly sensitive to the subtle changes of ligands (29).

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6319/1580/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S11
Tables S1 and S2

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MIGRATION

Mass seasonal bioflows of high-flying insect migrants

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Migrating animals have an impact on ecosystems directly via influxes of predators, prey, and competitors and indirectly by vectoring nutrients, energy, and pathogens. Although linkages between vertebrate movements and ecosystem processes have been established, the effects of mass insect “bioflows” have not been described. We quantified biomass flux over the southern United Kingdom for high-flying (>150 meters) insects and show that ~3.5 trillion insects (3200 tons of biomass) migrate above the region annually. These flows are not randomly directed in insects larger than 10 milligrams, which exploit seasonally beneficial tailwinds. Large seasonal differences in the southward versus northward transfer of biomass occur in some years, although flows were balanced over the 10-year period. Our long-term study reveals a major transport process with implications for ecosystem services, processes, and biogeochemistry.

Latitudinal migrations of vast numbers of flying insects, birds, and bats (1–7) lead to huge seasonal exchanges of biomass and nutrients across the Earth’s surface (8–11). Because many migrant species (particularly insects) are extremely abundant (1, 5), seasonal migrations may profoundly affect communities through predation and competition while transferring enormous quantities of energy, nutrients, propagules, pathogens, and parasites between regions, with substantial effects on essential ecosystem services, processes, and biogeochemistry (8–11), and, ultimately, ecosystem function.

Latitudinal bird migrations are well characterized; for example, 2.1 billion passerines migrate annually between Europe and Africa (2), integrating multisensory navigational information (12), exploiting favorable winds and adopting adaptive

flight behaviors (13). By comparison, even though insect migration surpasses all other aerial migratory phenomena in terms of sheer abundance (1), latitudinal insect migration is largely unquantified, in particular for the majority of species that migrate hundreds of meters above the ground (5). Specialized radar techniques are required to study these high-flying insect migrants, as they are too small to carry transmitters or to be observed by any other means (14). Until now, radar studies have been aimed almost exclusively at quantifying migrations of relatively few nocturnal species of agricultural pests (3).

We quantified annual abundance and biomass of three size categories of diurnal and nocturnal insects migrating above an area of ~70,000 km² of the southern United Kingdom (Fig. 1A), between 150 and 1200 m above ground level (agl) (Fig. 1B), from 2000 to 2009 (15). Abundance and biomass values for medium (10 to 70 mg) and large insects (70 to 500 mg) (referred to collectively as “larger insects”) were calculated from measurements of >1.8 million individuals (table S1) detected by vertical-looking entomological radars (VLRs) located in the southern United Kingdom (Fig. 1A). The VLRs provide a range of information—including body mass, flight altitude, aerial density, displacement speed, displacement direction, and flight heading—for all individual

insects of >10-mg body mass that fly through the vertically pointing beam within the altitude range of 150 to 1200 m agl (14). Annual abundance and biomass values for larger insects migrating over the study area were extrapolated from the aerial densities and body masses recorded above the VLR locations (15). The third size category, small insects (<10 mg), are not sampled by VLRs, and so abundance and biomass data were calculated from aerial netting samples (16) taken ~200 m agl near one of the radars (Fig. 1A) and extrapolated to the study area (15). Larger diurnal migrants are predominantly beneficial species, including hoverflies, ladybeetles, carabid beetles, and butterflies (14–17), and the most abundant small day-fliers are cereal aphids (16). The commonest larger nocturnal insects are lacewings and noctuid moths (14, 16), whereas Diptera constitute the majority of the small nocturnal insects (16).

An annual mean of 3.37 trillion insects (range 1.92 to 5.01 × 10¹²) (Fig. 1C and table S2) migrated high above the study region, comprising 3200 tons of biomass (fig. S1 and table S3), and >70% of that biomass was from migration that occurred during daytime (Fig. 1C and table S2). Numerically, >99% of individuals were small insects; although the 15 billion medium and 1.5 billion large insects made up only 0.4% and 0.05% of the annual abundance (table S2), they accounted for a substantial proportion of the biomass: 12% (380 tons) and 7% (225 tons), respectively (table S3).

By analyzing 1320 daytime “mass migrations” (15) involving 1.25 million VLR-detected insects and 898 nocturnal mass migrations involving 126,000 insects (table S1), we characterized migration directions of the larger insects during “spring” (May to June), “summer” (July) and “fall” (August to September) (fig. S2 and table S4). Although high-altitude winds blew consistently toward the northeast or east in all three seasons (Rayleigh tests; daytime: spring, 60°; summer, 66°; fall, 84°; nighttime: spring, 69°; summer, 81°; fall, 101°) (Fig. 2A and table S5), mass migrations of larger insects did not simply move with the prevailing southwesterly winds. During the spring, mass migrations were consistently toward the north (Rayleigh tests; daytime: medium, 333°; large, 329°; nighttime: medium, 349°; large, 349°) (Fig. 2A), and this indicates that migration occurred on winds with a significantly more southerly component than prevailing winds (Watson-Wheeler tests; *P* < 0.0001 in all cases) (table S5).

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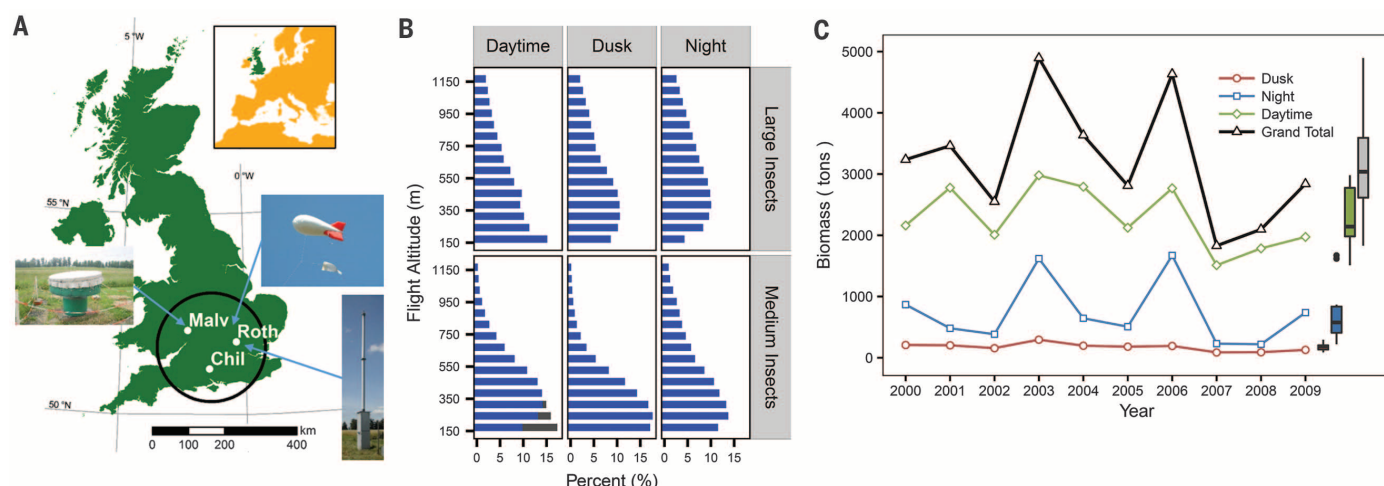


Fig. 1. Monitoring migration intensity above the southern United Kingdom. (A) The intensity and direction of high-altitude insect migration through the atmosphere 150 to 1200 m above ground level (agl) was measured over a 70,000-km² region of the southern United Kingdom (black circle) under continual surveillance from vertical-looking radars (VLR, left inset) at three locations (white dots); the aerial insect fauna were sampled by balloon-supported aerial netting at 200 m agl (center-right inset) and 12-m-high

suction traps (bottom-right inset). (B) Vertical profiles of larger insect (>10 mg) migration intensity over the sampling range of the VLRS. (C) Annual totals of insects migrating above the study region, during daytime, dusk, and night, as well as combined for the whole 24 hours. Lines represent annual totals; in the box plots, the central bar represents the median, boxes represent the interquartile range (IQR), whiskers extend to observations within ± 1.5 times the IQR and dots represent outliers.

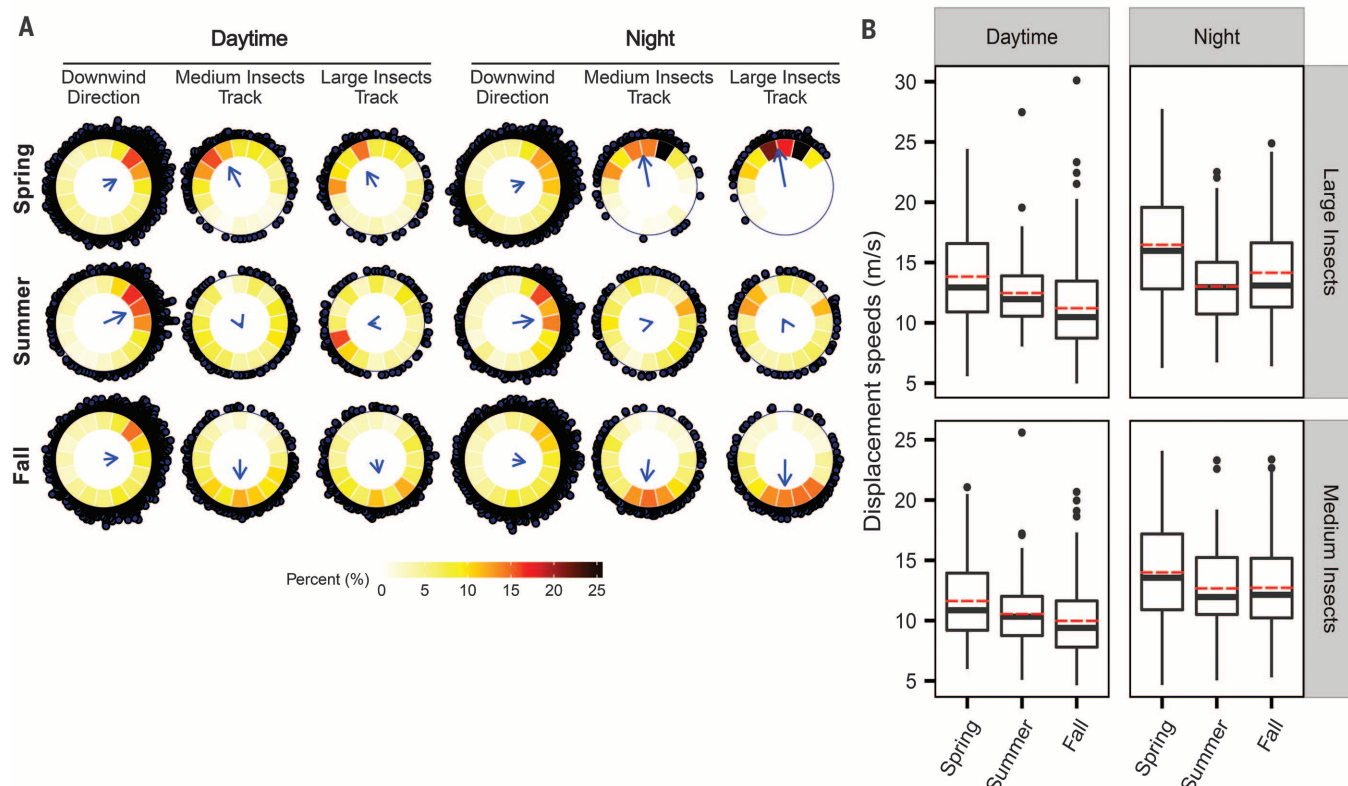


Fig. 2. Migratory directions and speeds of high-flying larger insects. (A) Despite prevailing winds blowing toward the northeast in all seasons, migratory tracks and headings occurred predominantly in seasonally beneficial directions in spring and fall but were randomly directed in summer. Small black circles on the periphery of the circular histograms represent mean directions of individual mass migrations, and the color bar indicates the percentage of migrations in

each 22.5° bin. The bearing of the blue arrow indicates the overall mean direction, and arrow length represents the circular resultant length (r). (B) Migrants achieved fast displacement speeds. Solid black bars represent medians, dashed red lines represent means, boxes represent the IQR, whiskers extend to observations within ± 1.5 times the IQR, and dots represent outliers.

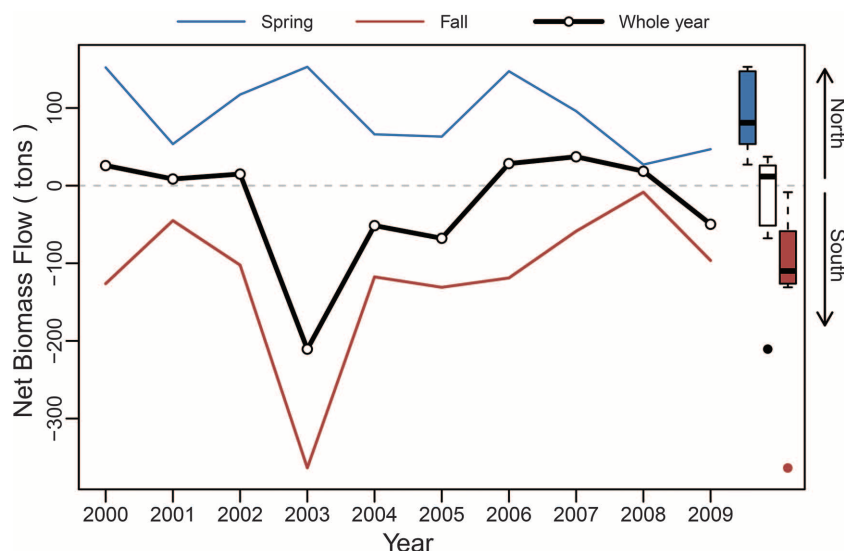


Fig. 3. Annual patterns of net directional migration. The net flow of biomass of larger insects above the study region, in spring, fall, and the whole year. Negative values indicate a net southward movement; positive values indicate a net northward movement. In the box plots, central bars represent median values, boxes represent the IQR, whiskers extend to observations within ± 1.5 times the IQR, and dots represent outliers.

Summer mass migrations were randomly directed (Rayleigh tests; $P > 0.05$ in all cases) (Fig. 2A and table S5), this indicated an absence of wind selectivity. By contrast, fall mass migrations were consistently directed toward the south (Rayleigh tests; daytime: medium, 174° ; large, 159° ; nighttime: medium, 181° ; large, 180°) (Fig. 2A), and this indicates active selection of winds with a significantly more northerly component than the prevailing winds (Watson-Wheeler tests; $P < 0.0001$ in all cases) (table S5). These relationships indicate preferred movement directions during the spring and fall, as well as selection of days and nights with favorably directed tailwinds. Seasonally beneficial migration directions have been previously reported in a few species of large insects, notably pest noctuid moths (3, 14, 18), but our findings demonstrate the ubiquity of such movements among a diverse array of insect migrants. Because small insects fall below the VLR's detection threshold (14) and, thus, their tracks cannot be directly measured, we used aphid migration intensity as representative of all small insect migration (fig. S3) by analyzing wind directions associated with mass migrations of aphids (15). We found that aphid migration directions closely match prevailing wind directions, i.e., toward the northeast in all seasons (fig. S4), and, therefore, conclude that these small insects do not have mechanisms for selecting seasonally beneficial winds.

The greatest amount of variation in the aerial density of diurnal migrants was explained by surface meteorological conditions associated with fine weather (table S6): Migration intensity was greatest on warm days (fig. S5A) with moderate to high surface heat flux (fig. S5B) and low surface wind speeds (fig. S5C). However, the strong relationship with fine weather does not explain the directed movements of the larger insects in spring and fall. Models indicated that during

these seasons (but not summer), surface wind direction was also correlated with migration intensity, with high densities associated with southerly winds in the spring and northerly winds in the fall (table S6). Surface and high-altitude daytime wind directions were strongly correlated in all seasons (tests for T-linear association; $P < 0.001$ in all cases) (fig. S6) but not at night (18). Thus, surface wind direction provides a reliable cue regarding the suitability of winds aloft for diurnal migrants at take-off but not for nocturnal migrants, which must use other methods for assessing high-altitude wind direction (19). The ubiquity of tailwind selectivity in such a diverse group indicates that compass mechanisms must be universal in larger insect migrants.

If high-flying insects have a compass sense, one would predict that, in addition to selecting a favorable tailwind, they would also orientate in the seasonally beneficial direction and, thus, actively contribute to their wind-assisted displacement. Such "common orientation" was indeed highly prevalent in the larger insects (table S1), and headings were close to tracks (fig. S7): northward in the spring and southward in the fall (table S7). The close correspondence between headings and tracks signifies that larger insects added much of their self-powered airspeed to the wind vector and thus achieved rapid displacement speeds [10 to 16 m/s (36 to 58 km hr $^{-1}$)] (Fig. 2B and table S7). A flight duration of 4 hours could therefore result in transport over >200 km, and during spring and fall, this transfer of biomass and nutrients occurs in predictable directions.

What are the implications of this high-altitude insect movement? Insect bodies are typically composed of 10% nitrogen and 1% phosphorus by dry weight (20), and as such, they represent a rich source of nutrients that can be limiting for plant productivity (17). The 3200 tons of biomass

moving annually above our study region contains $\sim 100,000$ kg of N and 10,000 kg of P, representing 0.2% of the surface deposits of N and 0.6 to 4.7% of P from the atmosphere, and making up 5.78×10^{12} Joules of energy (15). To put these seasonal movements in context, the annual airborne insect biomass >150 m above the southern United Kingdom is 4.5 times the 2.2 billion (700 tons) of bogong moths (*Agrotis infusa*) that migrate to the Australian Alps every summer (7, 9), 7.7 times the 30 million songbird migrants (415 tons) that depart the United Kingdom for Africa each fall (table S8), and >40 times the 150 million monarch butterflies (75 tons) that migrate between eastern North America and Mexico (14).

If the spring and fall movements documented here perfectly counterbalance each other, there would be no net annual exchange of energy and nutrients, and the principal consequences would be the exchange of genes, pathogens, and parasites. Over the 10-year study, we found that net northward spring movements of larger insects were almost exactly cancelled out by net southward fall movements; however, on an annual basis, the net flux could be up to 200 tons greater in either direction (Fig. 3). Such insect movements represent an underappreciated mechanism for redistributing nutrients and energy, and if the densities observed over southern United Kingdom are extrapolated to the airspace above all continental landmasses, high-altitude diurnal insect migration represents the most important annual animal movement in terrestrial ecosystems, comparable to the most significant oceanic migrations (21). Given the worrying declines in many migrants (8), developing global surveillance techniques (6) for long-term observation and prediction of the impacts of mass aerial migrations at such macrosystem scales (22) should be a priority for ecologists.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6319/1584/suppl/DC1
Materials and Methods
Figs. S1 to S8

Tables S1 to S8
References (23–49)

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BRAIN RESEARCH

Active cortical dendrites modulate perception

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There is as yet no consensus concerning the neural basis of perception and how it operates at a mechanistic level. We found that Ca^{2+} activity in the apical dendrites of a subset of layer 5 (L5) pyramidal neurons in primary somatosensory cortex (S1) in mice is correlated with the threshold for perceptual detection of whisker deflections. Manipulating the activity of apical dendrites shifted the perceptual threshold, demonstrating that an active dendritic mechanism is causally linked to perceptual detection.

Recent studies in awake rodents indicate that dendritic Ca^{2+} activity in L5 cortical pyramidal neurons is elevated during cognitive processes (1–4). These studies are in line with the proposal that the Ca^{2+} electrogenic properties of the apical dendrites of pyramidal neurons (5, 6) amplify the effects of feedback inputs [apical amplification (7)] to superficial cortical layers (8, 9). There is evidence for the crucial role of feedback to primary sensory regions in perceptual processes (11, 12), but it still remains to be demonstrated experimentally that perception depends on a dendritic mechanism. We reasoned that the decisiveness of this mechanism could be tested by examining dendritic Ca^{2+} activity around the perceptual threshold that corresponds to the transition from subliminal to liminal perception in humans (13). The apical amplification hypothesis predicts that dendritic Ca^{2+} activity in a subset of neurons correlates to this transition, leading to the detection of stimuli (Fig. 1A). To test this experimentally, we used a whisker-based tactile detection task in mice (10) combined with two-photon Ca^{2+} imaging of pyramidal apical dendrites in S1. Additionally, we investigated the causal relationship between dendritic Ca^{2+} and perceptual detection by testing whether manipulating dendritic Ca^{2+} currents alters detection.

Animals were first trained to report whisker deflections by licking to obtain water rewards (Fig. 1, B and C). The C2 whisker was selectively stimulated using a magnetic coil (fig. S1). The mice learned the task (>80% correct “hit” re-

sponses), with low false alarm rates (i.e., the licking rate at zero stimulus intensity), within 2 weeks, at which point we determined the psychometric function for whisker stimulation by using a range of intensities above and below the perceptual detection threshold (Fig. 1D). The perceptual detection threshold for individual animals was determined by fitting the psychometric function with a sigmoid function (14) (Fig. 1E). The behavioral thresholds remained at the same level across sessions over days (Fig. 1F). The delay between the whisker deflection and an animal’s reaction (first lick) in “hit” trials was shorter in the trials of salient stimuli than in threshold stimuli (Fig. 1G).

We simultaneously performed fast-scanning two-photon Ca^{2+} imaging from apical dendrites of L5 neurons in the C2 barrel column in primary somatosensory cortex (S1) (Fig. 1H). In each field of view (175 by 175 μm), 98.1 ± 17.8 apical dendrites were identified at 200 to 300 μm below the pia (mean $\pm$ SD, $n = 8$ sessions from 8 mice). During the behavior, large Ca^{2+} transients were observed in some apical dendrites after whisker deflections (Fig. 1I). To investigate the relationship between dendritic activity and perceptual behavior of the animal, Ca^{2+} responses were determined for seven different stimulus intensities around the behavioral threshold (Fig. 1I, vertical columns). We compared the activity in “lick” trials (Fig. 1I, lower rows) to “no-lick” trials (upper rows) and averaged the responses over all trials (bottom traces).

How does dendritic Ca^{2+} relate to behavior for this whisker detection task? To examine this, we plotted the dendritic Ca^{2+} response as a function of stimulus intensity—i.e., the neurometric function—versus detection probability as a function of stimulus intensity—i.e., the psychometric function (Fig. 2A, right). In some cases, the increase in dendritic Ca^{2+} closely followed the behavioral performance of the animal (Fig. 2A).

Interestingly, in a smaller fraction of cases, we found the opposite: dendritic Ca^{2+} anticorrelated with stimulus strength (Fig. 2A), which may be indicative of a parallel coding scheme such as predictive coding (15). The correlation of dendritic Ca^{2+} to behavior was quantified using a similarity index (SI) (16) and compared to chance level by shuffling the stimulus intensities of the original data (Fig. 2B). In 34% (267 of 785) of cases, the SIs deviated from chance. The fraction of dendrites responding to salient whisker deflections was greater in mice engaged in the task than naïve (untrained) mice (fig. S2).

We also investigated the discriminability of dendritic Ca^{2+} by evaluating the behavioral outcome (hit or miss) versus the Ca^{2+} response near the threshold for behavior—i.e., the discrimination index (DI) based on a receiver operating characteristic (ROC) analysis (17) (Fig. 2C) (also see methods in the supplementary materials). In 22% (173 of 785) of dendrites, the dendritic Ca^{2+} near threshold alone could be used to predict the behavior with prolonged increases in Ca^{2+} signals in “lick” trials, but not in “no-lick” trials (Fig. 2D, *DI > 0, $P < 0.05$; and Fig. 2E, left). Again, in some neurons (68 of 785), the behavior was predicted by a decrease in dendritic Ca^{2+} (Fig. 2D, *DI < 0, $P < 0.05$; and Fig. 2E, right). In another group of neurons with high SIs and low DIs, evoked Ca^{2+} signals increased with increasing amplitudes of whisker deflection but were independent of behavioral outcome, suggesting that these neurons coded for information orthogonal to perceptual detection (Fig. 2E, middle). We characterized the temporal and spatial features of perception-relevant dendrites (DI’s $P < 0.05$). The timing of the dendritic Ca^{2+} increases for high stimulus strengths was faster than that for near-threshold stimuli, and the dendritic Ca^{2+} mostly preceded the behavior (Fig. 2F and fig. S3). We found a small but significant relationship between the physical location of the dendrites and their DI (Fig. 2, G and H).

Activation of dendritic Ca^{2+} channels results in enhanced firing of individual L5 pyramidal neurons in vivo (18). We monitored the firing activity of L5 neurons while the animal performed the whisker detection task (Fig. 3A). The results of 40 cells recorded from L5 (depth, 577.8 to 774.0 μm below the pia) in behaving animals were pooled. Mean firing rates were 11.1 ± 14.2 Hz, ranging from 0.68 to 63.1 Hz (mean $\pm$ SD, $n = 40$ cells from 10 mice). In a fraction of neurons, perceived whisker stimuli increased firing activity, resulting in positive correlations between neurometric and psychometric functions (Fig. 3B). As seen in dendritic Ca^{2+} activity, we also observed some cells for which the firing activity

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Fig. 1. Behavior perceptual detection threshold for a whisker detection task. (A) Apical amplification (AA) hypothesis (7) in a sensory detection paradigm. Above the perceptual detection threshold, in addition to feed-forward information (blue arrows), pyramidal neurons in primary sensory cortex receive long-range feedback (FB) (red arrow) inputs from other cortical regions arriving at distal tuft dendrites. Active dendritic Ca^{2+} currents conjugate early sensory with feedback information (6), leading to sensory perception. (B) (Top) Behavioral task design. (Bottom) Deflection angle of whisker is proportional to stimulus intensity. Mean amplitude (color) plotted with individual data (gray) ($n = 7$ mice) (see also fig. S1). (C) Stimulus paradigm (left) and table of behavioral outcomes classified into four categories—i.e., hit, miss, false alarm (FA), and correct rejection (CR) (right). (D) (Top) Raster plot of lick events (gray) in 357 trials from a single session sorted by intensity of the presented stimulus. Rewarded (hit) licks marked in blue. (Bottom) Histograms of response timings of first lick at different stimulus intensities. (E) Psychometric function for the behavioral performance shown in (D). (F) Psychometric functions over 6 days, obtained from the same mouse. (G) (Left) Reaction times for stimuli above thresholds plotted as a function of stimulus intensity ($n = 15$ sessions from 15 mice). (Right) Reaction times for near-threshold stimuli and salient (maximum) stimuli. (H) Two-photon Ca^{2+} imaging from the apical dendrites of L5 neurons expressing GCaMP6s (left) in the C2 barrel column (bottom right). (Inset) Horizontal imaging plane (216 μm from pia). (I) Ca^{2+} signals in an apical dendrite marked in (H) during the detection task organized according to increasing stimulus intensity (columns) and trials (rows), separated based on the behavioral response (“lick” or “no-lick”) by a blank row. (Bottom) Average and SEM of Ca^{2+} responses for all trials of a given stimulus intensity.

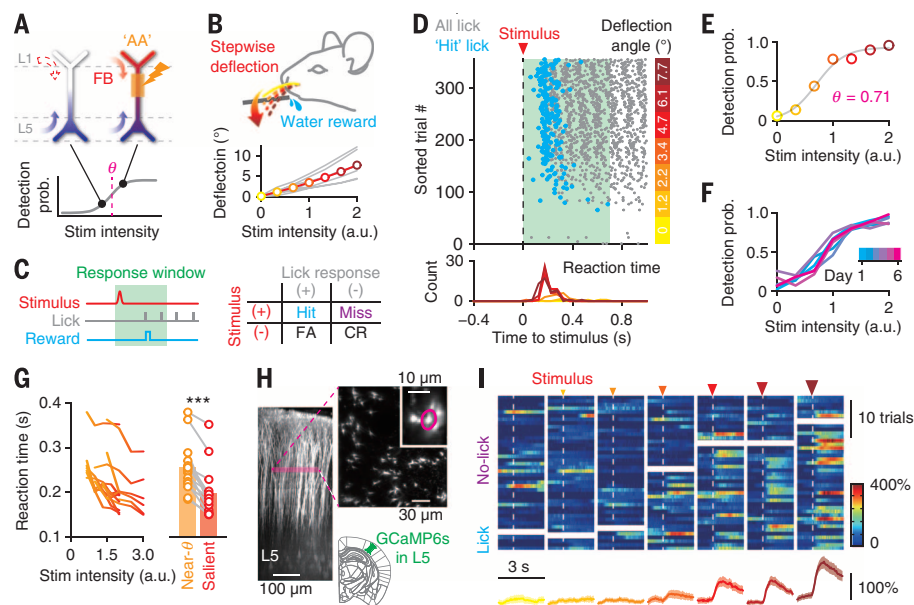
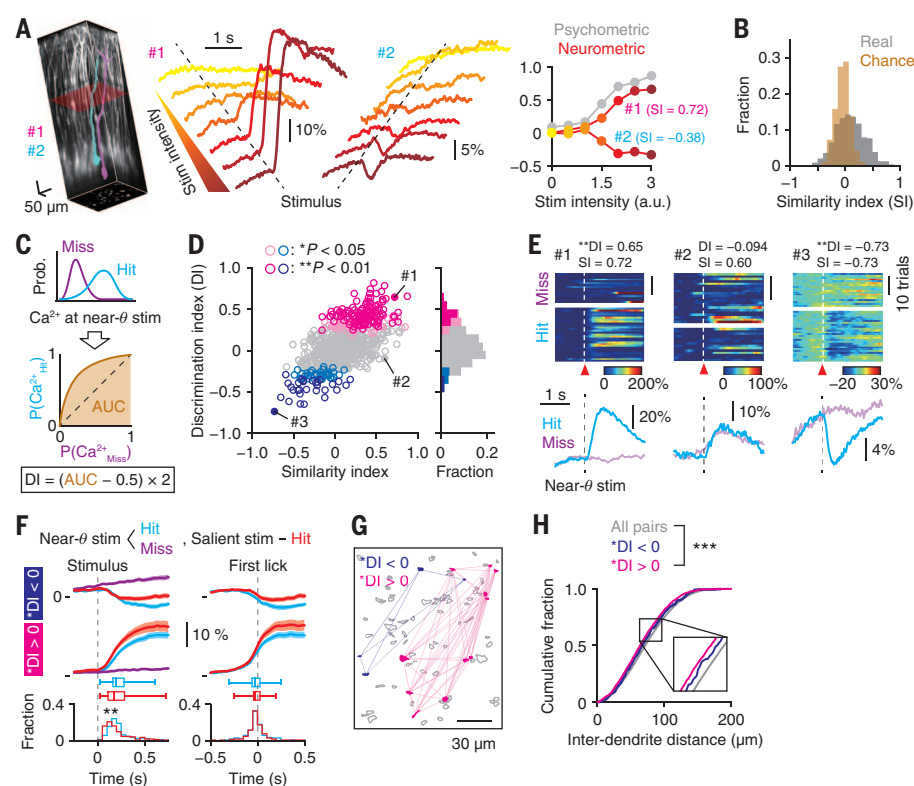


Fig. 2. Apical dendritic Ca^{2+} activity during the perceptual detection task. (A) (Left) Three-dimensionally reconstructed apical dendrites (#1 and #2) in the same field of view. Imaging plane is shown in red. (Middle) Averaged Ca^{2+} responses to various stimulus intensities from the two different apical dendrites. (Right) Neurometric functions of the dendritic Ca^{2+} activities compared with behavioral performance (psychometric function). (B) Correlation between neurometric and psychometric functions evaluated by the similarity index (SI) based on Euclidean distance. Distribution of SIs from real data (gray) compared with chance level (brown). (C) Schematic of ROC analysis (17): The discrimination index (DI)—the likelihood that dendritic Ca^{2+} activity predicted the animal's detection of near-threshold stimuli—estimated based on the area under the ROC curve (AUC). (D) (Left) Dendritic responses characterized by SIs and DIs. Dendrites with high discriminability shown in color. Statistical significance of DI was computed by a permutation test. (Right) Histogram of DIs. (E) (Top) Examples of individual dendritic Ca^{2+} responses to near-threshold stimuli for the three cases in (D). (Bottom) Average Ca^{2+} activity during hit (blue) and miss (purple) trials. (F) (Top) Timing of the Ca^{2+} response to near-threshold and salient stimuli in discriminatory dendrites ($*P < 0.05$) aligned by the onsets of the stimuli (left) or by the timing of an animal's first (rewarded) licks (right). Average traces shown with SEM ($n = 173$ dendrites for $*DI > 0$, $n = 68$ dendrites for $*DI < 0$). (Bottom) Histograms of onset timings of Ca^{2+} responses in dendrites with $*DI > 0$ to near-threshold and salient stimuli in hit trials (stimulus-aligned, $**P = 9.7 \times 10^{-3}$; first lick-aligned, $P = 1.0$, Kolmogorov-Smirnov test). (G) Spatial distribution and interdendritic distance of imaged dendrites (outlined) in a single field of view with discriminatory dendrites denoted by color. (H) Cumulative fraction of dendritic pairs as a function of distance between imaged dendrites ($n = 39,232$ pairs, gray), dendrites with $*DI > 0$ ($n = 2456$ pairs, magenta), and dendrites with $*DI < 0$ ($n = 319$ pairs, blue) (all pairs versus $*DI > 0$, $***P = 4.2 \times 10^{-14}$, Kolmogorov-Smirnov test).



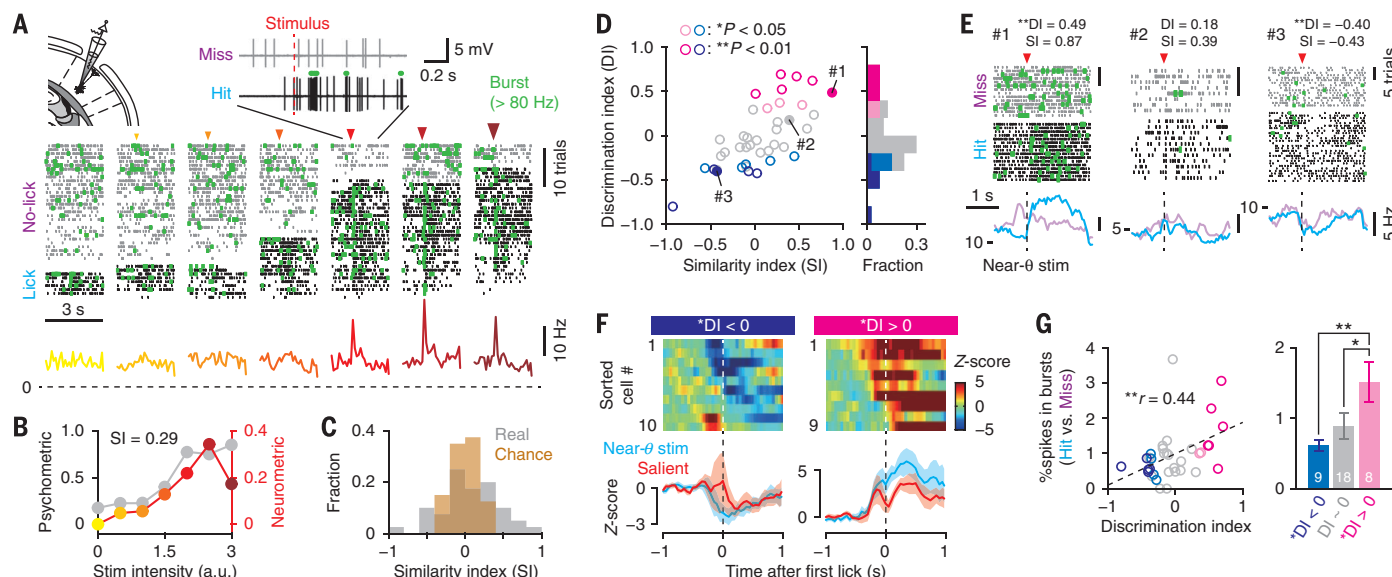


Fig. 3. Firing behavior of L5 neurons during the perceptual detection task. (A) (Top) Juxtacellular recording from behaving animals. (Middle) Raster plot of the firing activity of an L5 neuron in the C2 barrel column. Green dots in (A) and (E) indicate spikes in burst events (>80 Hz). (Bottom) Peristimulus spike time histograms (PSTHs). (B) Neurometric function of the neuron in (A) compared with the psychometric function. (C) Distribution of similarity indices (SIs) between neurometric and psychometric functions. (D) Firing responses of recorded neurons were characterized by SI and DI, as in Fig. 2D. (E) Example raster plots and PSTHs of firing responses of L5 neurons to near-threshold

whisker stimuli for the three cases in (D). (F) (Top) Normalized firing responses to near-threshold and salient stimuli in neurons with high discriminability (DI's $*P < 0.05$) aligned by the timing of behavioral reaction (first lick). (Bottom) Average PSTHs shown with SEM. (G) (Left) The proportion of spikes in bursts [i.e., green versus black/gray dots in (E)] for near-threshold stimuli in hit trials relative to miss trials as a function of DI (Pearson's correlation coefficient $r = 0.44$, $**P = 8.7 \times 10^{-3}$). (Right) Average proportional bursting for hit versus miss, shown with SEM for discriminatory neurons ($*DI < 0$, $*DI > 0$) and nondiscriminatory neurons ($DI \sim 0$) ($**P < 0.01$, $*P < 0.05$, Dunn's test after Kruskal-Wallis test).

negatively correlated to animal behavior. In 38% (15 of 40) of recorded cells, firing activity correlated (or anticorrelated) beyond chance to animal behavior (Fig. 3C). The same ROC analysis performed on dendritic Ca^{2+} (Fig. 2C) was used to determine the DI for the likelihood that a change in firing could predict the animal's behavior when near-threshold stimuli were given (Fig. 3, D and E). About 23% (9 of 40) of cells increased their firing activity to near-threshold stimuli preferentially in "hit" trials, whereas 25% (10 of 40) decreased their firing activity (Fig. 3D). In most cells that significantly correlated to the animal's behavior (DI's $P < 0.05$), the firing change preceded the licking reaction of the animal (Fig. 3F) (11 out of 19 neurons). The burstiness of firing correlated to discriminability (Fig. 3G), suggesting that burst firing is more salient for perceptual detection (19). However, burst firing was less predictive of behavior than spike rate alone. In summary, changes in firing corresponding to perceptual detection occurred in a similar proportion of L5 pyramidal neurons as observed with dendritic Ca^{2+} activity.

Our data show that dendritic Ca^{2+} signals are correlated to animals' perceptual behavior. Indeed, the timing between dendritic Ca^{2+} activity and licking behavior suggests a causal relationship (Fig. 2F and fig. S3). To investigate causality, we manipulated dendritic activity pharmacologically and optogenetically during the task. The γ -aminobutyric acid type B (GABA_B)–receptor agonist baclofen suppresses dendritic Ca^{2+} spikes (fig. S4) (20). Here, local application of baclofen to superficial layers

profoundly suppressed ongoing Ca^{2+} activity in apical tufts in awake animals (Fig. 4A). On the other hand, application of baclofen in vivo does not affect sensory-evoked subthreshold activity in L5 neurons (18). Importantly, baclofen also strongly shifted the animals' behavioral responses to higher stimulus values (Fig. 4B, left, and fig. S6B) with the largest changes occurring at the perceptual detection threshold (Fig. 4B, right). Saline had no effect on perceptual detection (fig. S6, E to I).

We expressed a chloride-conducting channelrhodopsin (iChloC) (27) in L5 neurons in the C2 barrel column. Brief illumination with blue light in the superficial cortical layers induced a prolonged suppression of Ca^{2+} activity in the apical dendrites of L5 neurons expressing iChloC (Fig. 4C). Ex vivo experiments confirmed that depth-calibrated 470-nm light used in vivo effectively blocked Ca^{2+} spikes in apical dendrites without any effects on somatic excitability (fig. S5). Inactivating the superficial dendrites with iChloC also caused a significant impairment in the animals' detection probability of threshold stimuli (Fig. 4D).

We took advantage of an innate inhibitory circuit in the cortex known to down-regulate dendritic activity (22, 23), the dendrite-targeting somatostatin (SOM) inhibitory cells. This circuitry has been shown to gate dendritic excitability (4, 24). We therefore suppressed dendritic activity by activating SOM cells with channelrhodopsin-2 (ChR2) (Fig. 4E). Suppression of dendritic activity via SOM cell activation significantly increased the

perceptual detection threshold (Fig. 4F). All of the manipulations to down-regulate dendritic activity shifted the psychometric function to the right toward higher stimulus intensities but did not alter the gain (slope) nor the false alarm rate (fig. S6) (25).

Finally, we tested the consequence of up-regulating apical dendrites of L5 neurons on the animals' perceptual detection. The apical dendrites of L5 neurons expressing ChR2 were photostimulated using a fiberoptic cannula, which directed the 470-nm light specifically to superficial layers in a single barrel column. This spatially confined dendritic activation efficiently shifted the detection threshold toward lower stimulus intensities (Fig. 4H and fig. S6S). Importantly, reinforced activity of apical dendrites caused an increase in false alarm rate, indicating that dendritic activity could contribute to the intrinsic generation of sensory perception (fig. S6U). Together, these results suggest that the activity in apical dendrites of L5 neurons is causally linked to the perceptual behavior of the animal.

The results presented here show that a Ca^{2+} -dependent mechanism in the apical dendrites of L5 pyramidal neurons causally influences perceptual detection (6). They are consistent with recent studies showing that dendritic Ca^{2+} activity is correlated with behavior (1, 2, 4). Our data show that this dendritic mechanism is activated at the crucial transition from subliminal to liminal detection and can therefore be regarded as a neural correlate for perceptual detection. Four contrasting approaches that manipulated dendritic activity

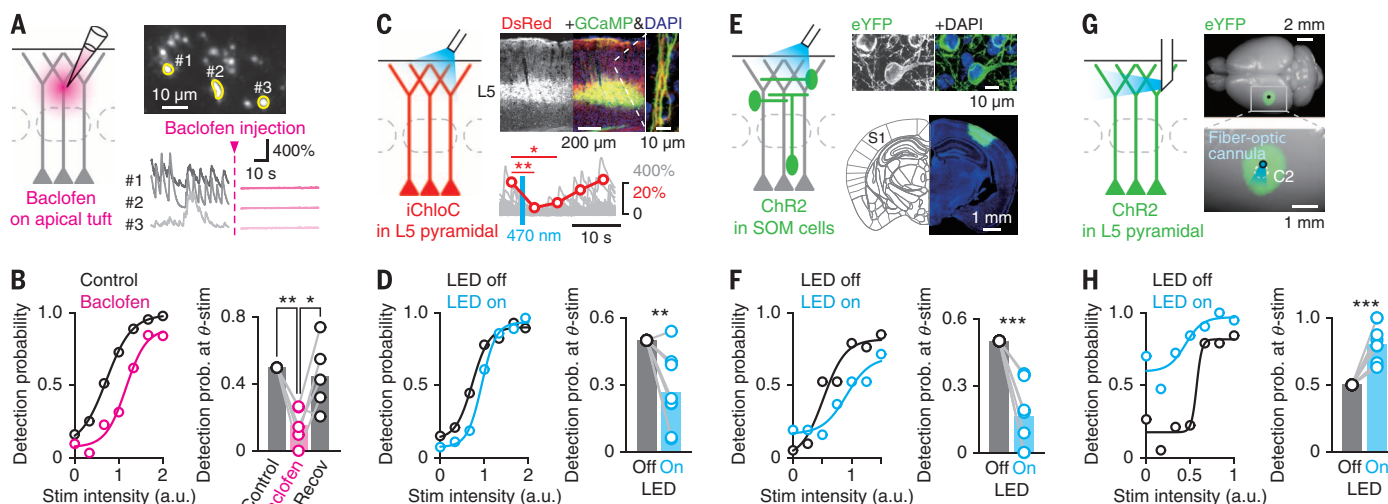


Fig. 4. Dendritic activity causally linked to perceptual detection threshold.

(A) (Left) Schematic diagram of local application of GABA_B-receptor agonist, baclofen, to superficial layers of C2 barrel column. (Right) Ongoing Ca²⁺ activity in apical dendrites of GCaMP6s-expressing L5 neurons before and after the baclofen application. (B) (Left) Psychometric curves with and without local baclofen application. (Right) Effect of baclofen on the detection probability at the threshold stimulus intensity compared with control conditions [$n = 5$ mice, $^{**}P < 0.01$, $^{*}P < 0.05$, paired t test with Bonferroni's correction after one-way repeated-measures analysis of variance (ANOVA)]. (C) (Left) Optogenetic inactivation of apical dendrites of L5 neurons expressing iChloC. (Right) Brief illumination with blue light followed by a prolonged suppression of Ca²⁺ activity (bottom, $n = 120$ traces from 10 dendrites, $^{**}P < 0.01$, $^{*}P < 0.05$, Dunnett's test after ANOVA) in

apical dendrites coexpressing iChloC and GCaMP6s (top). (D) (Left) Psychometric curves with [light-emitting diode (LED) on] and without (LED off) iChloC activation. (Right) Same as (B), with iChloC activation ($n = 8$ mice, $^{**}P = 5.8 \times 10^{-3}$, paired t test). (E) (Left) Controlling dendritic activity by activating dendrite-targeting SOM inhibitory cells. (Right) Specific expression of ChR2-eYFP in SOM cells in the C2 barrel column of S1. (F) Same as (B) and (D), with SOM-cell activation ($n = 7$ mice, $^{***}P = 9.2 \times 10^{-4}$, paired t test). (G) (Left) Optogenetic local activation of apical dendrites of L5 neurons expressing ChR2 using a fiberoptic cannula with a 45°-mirror tip. (Right) Expression of ChR2-eYFP in the C2 column and local photostimulation with the fiberoptic cannula. (H) Same as (B), (D), and (F), with ChR2 activation ($n = 9$ mice, $^{***}P = 2.1 \times 10^{-4}$, paired t test).

(both up and down) also significantly shifted the perceptual threshold in both directions (Fig. 4), suggesting a causal influence of apical calcium activity on perceptual detection.

The Ca²⁺ signals we recorded most likely represent the local activation of voltage-sensitive Ca²⁺ channels because single back-propagating action potentials (APs) and excitatory postsynaptic potentials (EPSPs) have little influence on apical dendritic Ca²⁺ signals (26, 27). Although we cannot completely rule out the possibility that some of the dendritic Ca²⁺ signals were caused by bursts of back-propagating APs generated by proximal synaptic input (28), the fact that suppression or up-regulation of the dendrites specifically altered the animal's behavior argues against this interpretation and for the conclusion that they resulted from dendritic Ca²⁺ spikes (2), which is also consistent with elevated burst firing (5).

It has been suggested elsewhere that apical amplification via dendritic Ca²⁺ currents relates to conscious perception (7, 29, 30). The evidence presented in this study points in this direction because the task used, simple sensory detection, is often used to investigate conscious perception in humans (13, 31, 32). However, it is difficult to establish a relationship between apical amplification and perceptual experience in rodents. It has recently been shown that transcranial magnetic stimulation can noninvasively suppress Ca²⁺ activity in pyramidal dendrites (33), which may make it possible to test this mechanism in humans in the future.

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SUPPLEMENTARY MATERIALS

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FISHERIES

Large benefits to marine fisheries of meeting the 1.5°C global warming target

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Translating the Paris Agreement to limit global warming to 1.5°C above preindustrial level into impact-related targets facilitates communication of the benefits of mitigating climate change to policy-makers and stakeholders. Developing ecologically relevant impact-related targets for marine ecosystem services, such as fisheries, is an important step. Here, we use maximum catch potential and species turnover as climate-risk indicators for fisheries. We project that potential catches will decrease by more than 3 million metric tons per degree Celsius of warming. Species turnover is more than halved when warming is lowered from 3.5° to 1.5°C above the preindustrial level. Regionally, changes in maximum catch potential and species turnover vary across ecosystems, with the biggest risk reduction in the Indo-Pacific and Arctic regions when the Paris Agreement target is achieved.

The effort required to achieve the Paris Agreement target of limiting global warming to 1.5°C relative to the preindustrial level appears to be substantial (1). Demonstrating the benefits of moving toward the Paris Agreement's target may encourage countries to commit more ambitious plans for reduction of greenhouse gas emissions and promote voluntary actions from private sectors (1, 2). Climate change is affecting marine biodiversity and ecosystem services such as food provision, posing substantial risk to the well-being of coastal communities (3–5). Quantifying the relationship between fisheries impacts and global surface warming (and thus cumulative carbon emissions) enables us to estimate the benefits of meeting the Paris Agreement target (Fig. 1).

We analyze the outputs from 19 Earth system models used in the Fifth Assessment of the Intergovernmental Panel on Climate Change that participated in the Coupled Models Intercomparison Project Phase 5 (CMIP5) under the Representative Concentration Pathway (RCP) 2.6 (strong mitigation) and 8.5 (high emission) scenarios (see supplementary materials). We evaluate the relationship between the projected global mean atmospheric surface warming between 1950 and 2100 and changes in oceanographic variables that drive changes in marine ecosystems (6, 7). The focus of our analysis is on the system of large marine ecosystems (fig. S2), which host more than 90% of the global catches and most of the diversity of exploited species. We find that global warming scales nearly linearly with ecosystem drivers at the sea surface averaged over all large marine ecosystems in all individual models under both RCP 2.6 and RCP 8.5 (6, 8) (Fig. 2; see supplementary materials). These drivers, including global mean sea surface temperature (SST), surface oxygen (O₂), and net primary production (NPP) integrated over the top

100-m depths of the ocean, are projected to change by $0.75 \pm 0.00^\circ\text{C}$ (coefficient of determination $R^2 = 0.99$, Fig. 2A), $-3.41 \pm 0.02 \text{ mmol}\cdot\text{m}^{-3}$ ($R^2 = 0.97$, Fig. 2C), and $-0.19 \pm 0.01 \text{ Pg C}\cdot\text{year}^{-1}$ ($R^2 = 0.26$, Fig. 2E), respectively, for every degree Celsius increase in global mean atmospheric surface temperature. Temperature and O₂ at sea bottom (Fig. 2, B and D), however, continue to change even when sea surface conditions are stabilized because of the relatively slow surface-to-deep ocean transport of heat and carbon.

We quantify the reduction in the impacts on marine fisheries from achieving the Paris Agree-

ment target globally, and regionally, using a Dynamic Bioclimate Envelope Model (DBEM; see supplementary materials). DBEM is a marine species distribution model that simulates the interactions between changes in ocean conditions, ecophysiology, population dynamics, dispersal, habitat productivity, and suitability, as well as fishing, in determining abundance and catches. With ocean warming, populations are projected to shift their distributions following gradients of habitat suitability (9), while changes in energy available from NPP for species' biomass production will also affect their potential catches (10, 11). DBEM is applied to 892 species of exploited marine fishes and invertebrates that are shown to be representative of total catches (see supplementary materials). Outputs from the DBEM include changes in abundance and catches driven by scenarios of ecosystem drivers obtained from the Earth system models. We use outputs from 3 of the 19 Earth system models that have the full set of ecosystem drivers under both RCP 2.6 and 8.5 scenarios, and that do not share the same ocean biogeochemical submodel (see table S1).

We express impacts to fisheries by changes in maximum catch potential (ΔMCP) (10) and turnover of exploited species ($\Delta\text{SppTurn}$) (12) (number of species newly occurring plus locally extinct relative to species richness in years 1950 to 1969) (see supplementary materials). MCP is a proxy for maximum sustainable yield, which is a commonly used reference point for fisheries management (see supplementary materials).

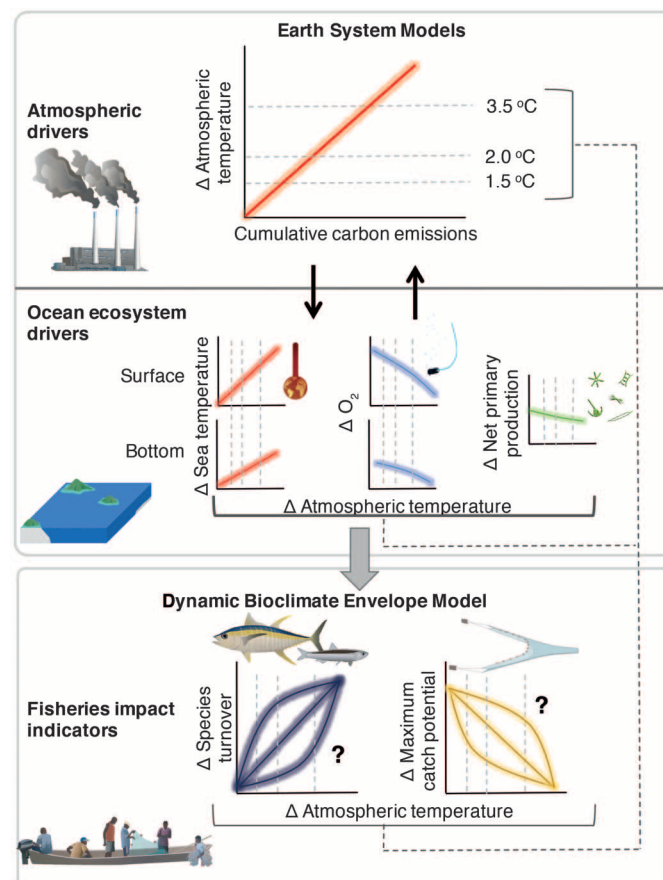


Fig. 1. Conceptual diagram explaining the framework of this study to establish ecologically relevant impact-related targets and the implications of such targets for understanding the benefits of meeting the Paris Agreement global warming target.

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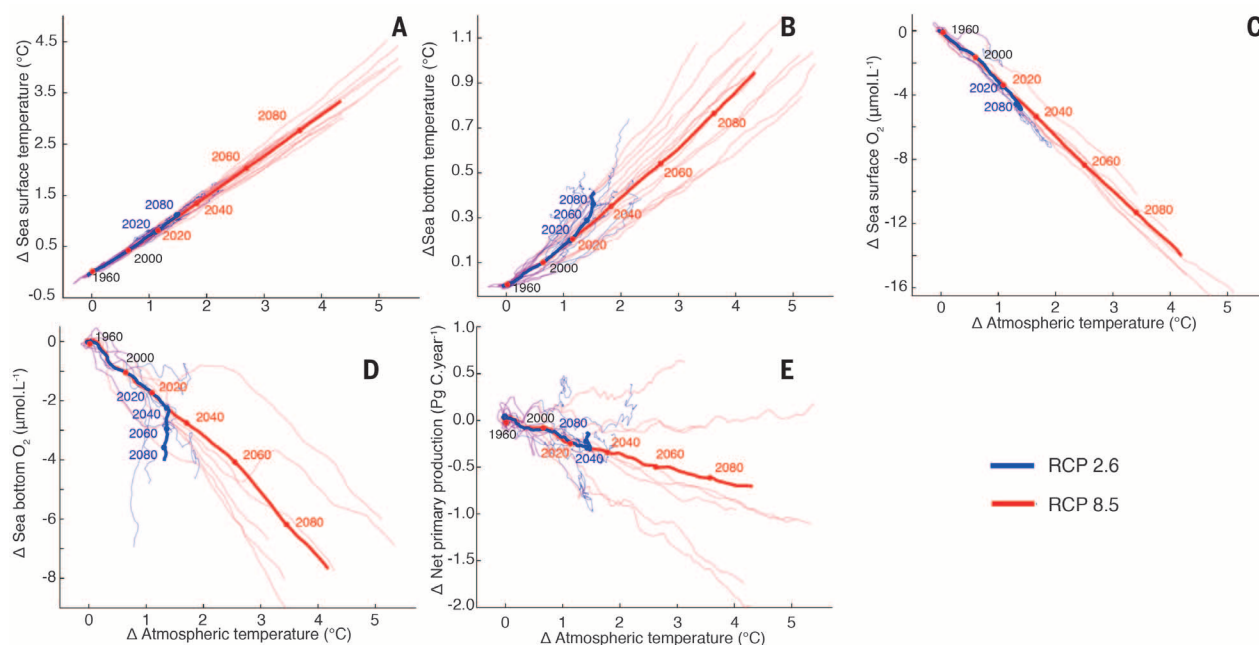
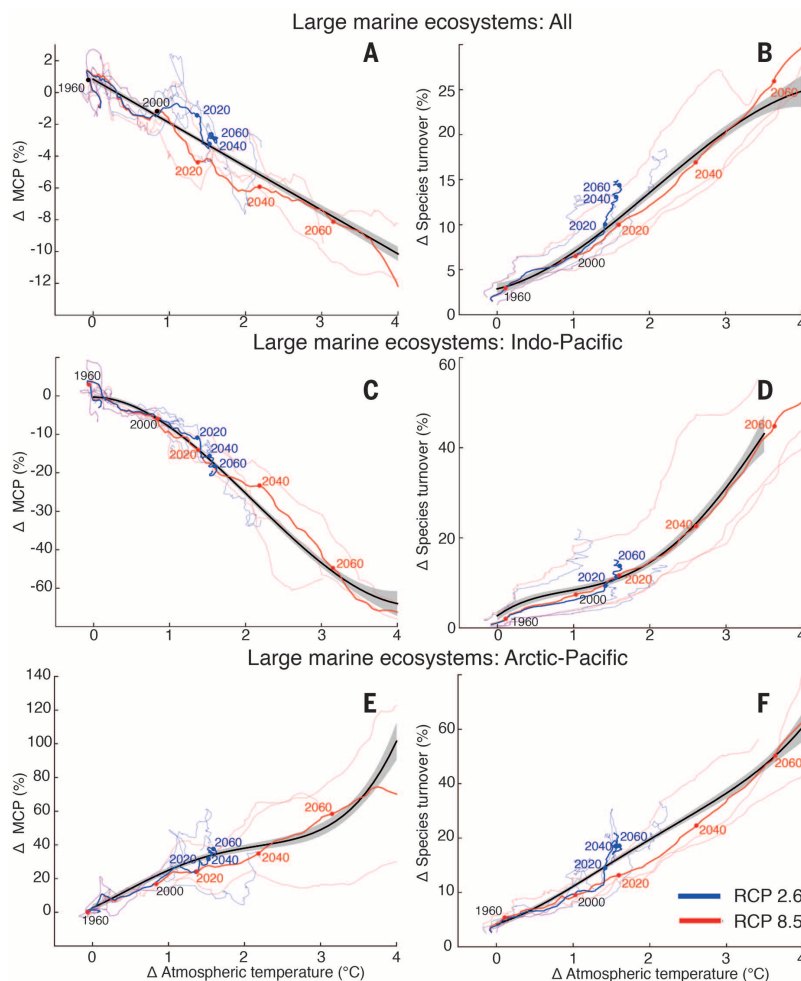


Fig. 2. (A to E) Scaling between global changes in marine ecosystem drivers and simulated surface air temperature relative to 1950 to 1969 (see supplementary materials). The thinner lines represent individual model projections, while the thicker lines represent multimodel averages. The time series are smoothed by a 10-year running mean.

Fig. 3. Projected global and regional ΔMCP (A, C, and E) and ΔSppTurn (B, D, and F) versus atmospheric surface warming relative to 1950 to 1969. The thinner lines represent individual model projections, while the thicker lines represent multimodel averages. The black lines and gray bands represent the selected regression model and their 95% confidence limits. The time series are smoothed by a 10-year running mean.



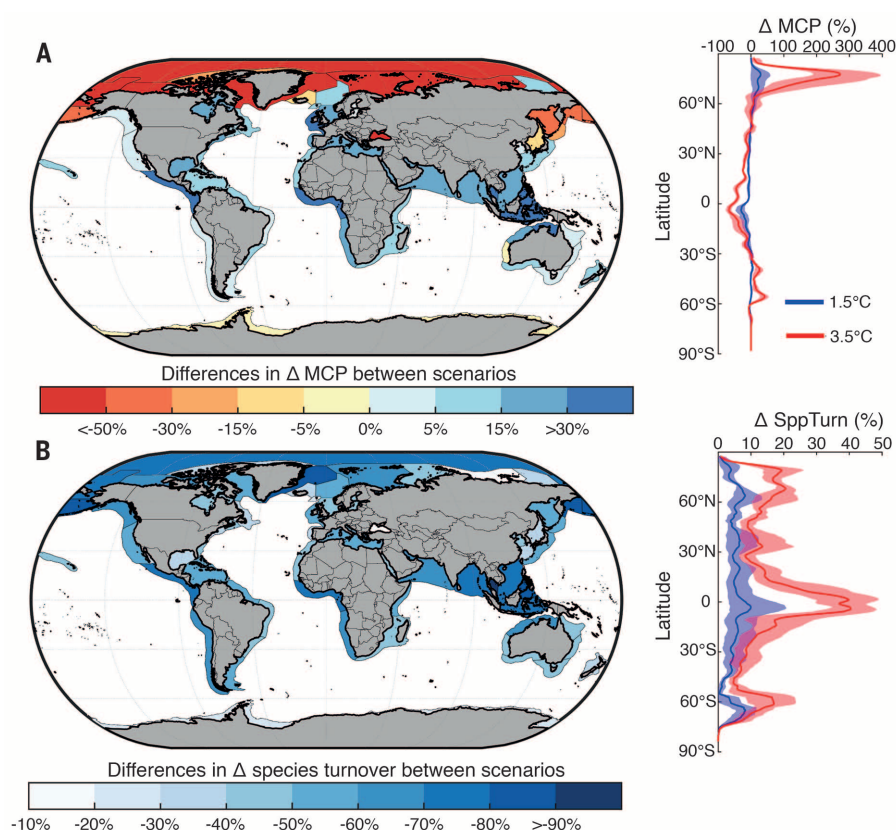


Fig. 4. Projected differences in Δ MCP (A) and Δ SppTurn (B) if global surface temperature were to be effectively limited from 3.5°C to 1.5°C relative to the preindustrial level. Gridded outputs are averaged by large ecosystem systems. The right panels are latitudinal zonal averages over the large marine ecosystems under the 3.5°C (red) and 1.5°C (blue) scenarios. Solid lines represent averages, while the bands represent maximum and minimum values across projections from the three Earth system models.

Projected Δ MCP integrated over all the large marine ecosystems scales negatively and nearly linearly with average atmospheric warming (Fig. 3A; see supplementary materials). The loss of catch potential is projected to be $2.8 \pm 0.15\% \text{ } ^\circ\text{C}^{-1}$ warming or $3.4 \times 10^6 \pm 0.37 \times 10^6$ metric tons $^\circ\text{C}^{-1}$, assuming a baseline global maximum catch potential of 1.23×10^8 metric tons (i.e., average of the top 10-year global annual catches since 1950, which is slightly higher than the average catches from 2001 to 2010; www.seaaroundus.org). Under 1.5°C global atmospheric warming relative to the preindustrial level, Δ MCP is projected to be $-2.5 \pm 0.2\%$ relative to the MCP at 0.27°C warming from preindustrial level (i.e., level of warming in the 1950s; see supplementary materials). In contrast, under 3.5°C global atmospheric warming relative to the preindustrial level (estimated the end of 21st-century warming level according to the published greenhouse gas emission reduction commitment by countries; see supplementary materials), we project a tripling of impacts from the 1.5°C target, with Δ MCP of $-8.0 \pm 0.34\%$.

The average species turnover (Δ SppTurn) across all the large marine ecosystems is projected to increase with global atmospheric warming (Fig. 3B). With average atmospheric warming of 3.5°C relative to the preindustrial level, species turnover is projected to be $21.6 \pm 0.33\%$ of the

average species richness between 1950 and 1969. However, if warming is restricted to 1.5°C, average species turnover is reduced to $8.3 \pm 0.05\%$.

Regional scaling between fisheries impacts and atmospheric warming is generally nonlinear in the tropics and Arctic, suggesting that the 1.5°C to 2.0°C global warming target represents a threshold above which substantial fisheries impacts are projected to occur. In the Indo-Pacific region (including the Bay of Bengal, Gulf of Thailand, South China Sea, and Sulu-Celebes Sea; see supplementary materials), Δ MCP is projected to be $-46.8 \pm 1.2\%$ under 3.5°C warming. However, Δ MCP is reduced to $-11.5 \pm 0.6\%$ and $20.2 \pm 0.6\%$ under 1.5°C and 2.0°C warming, respectively (Fig. 3C). Similarly, Δ SppTurn is projected to be $36.4 \pm 2.1\%$ under 3.5°C warming but decreases to $9.2 \pm 0.8\%$ and $12.1 \pm 0.8\%$ under 1.5°C and 2.0°C warming (Fig. 3D). In contrast, in the Pacific-Arctic (including the Bering Sea, Chukchi Sea, and Sea of Okhotsk), Δ MCP increases from $29.1 \pm 1.6\%$ to $55.0 \pm 3.9\%$ when global atmospheric temperature increases from 1.5°C to 3.5°C (Fig. 3E), whereas Δ SppTurn increases from $13.1 \pm 0.4\%$ to $30.5 \pm 1.0\%$ (Fig. 3F). Alternative patterns of scaling of fisheries impacts and average warming appear in some large marine ecosystems because of specific interactions between changes in ocean conditions and the

biological responses (see figs. S3 and S4 and tables S2 and S3). In the Norwegian Sea (figs. S3 and S4), for example, MCP increases initially with atmospheric warming through species gains and increasing abundance of exploited species, but then decreases at a high level of warming; the latter is driven by the decrease in projected NPP due to enhanced stratification and the onset of nutrient limitations in the Arctic (13). In the Antarctic region, the decrease and then increase in MCP under global warming are driven by the decrease in abundance of existing species, followed by the gain of lower-latitude species.

Climate-risk reduction for fisheries is projected to be the largest in tropical oceans under 1.5°C warming, partly because of the avoided local extinction (Fig. 4). Countries such as Ecuador, Costa Rica, Ghana, Thailand, the Philippines, and Indonesia, with their exclusive economic zones and fisheries in these large marine ecosystems, will benefit substantially from meeting the Paris Agreement. In contrast, the increase in habitat suitability and productivity in the Arctic Ocean corresponds to an increase in Δ MCP and Δ SppTurn. Particularly, the low baseline MCP in the high Arctic under current climate results in a large projected increase in MCP under warming. For mid-latitude regions, projected Δ MCP and Δ SppTurn are lower than in polar and tropical regions.

The nonlinear scaling of Δ MCP and Δ SppTurn is due to changes in environmental conditions beyond the species' physiological tolerance limits or when new suitable habitats become available (14, 15). Specifically, in the model, each species has its characteristic temperature preferences and limits, and other ocean conditions such as oxygen content and primary production would indirectly modify that. Species will become locally extinct if temperature goes beyond those limits (see supplementary materials). In the tropics, temperature and ocean conditions are projected to exceed what the species have experienced in the last century (16, 17). As the tropics generally represent one end of the environmental limits (17), a large proportion of these species are projected to become locally extinct if they cannot physiologically adapt to changes beyond these limits (17, 18). In contrast, warming and the rapid retreat of sea ice in the Arctic Ocean open up new habitats for marine species for sub-Arctic and temperate species to expand into (19, 20). Polar species, however, have evolved narrower temperature tolerance limits because of the historically stable climatic conditions relative to the temperate environment, rendering them more sensitive to ocean warming (9, 21). In addition, species turnover is suggested to cause indirect impacts through alteration of ecosystem structure and food web interactions (18, 22). These impacts pose social and economic challenges to fisheries—for instance, through decrease in revenues, livelihood, and food availability (3, 4).

The patterns of our projections generally agree with observations and model simulation studies on climate change impacts on marine species (18, 21, 23). For example, the decrease in potential

catches of Atlantic cod in the Gulf of Maine driven partly by rapid ocean warming has contributed to the collapse of the cod fisheries (24). In the Gulf of Mexico, turnover of fish species in sea-grass assemblages as a result of species gains and losses between the 1970s and 2000s have been reported (25). In the Barent Sea, recent warming since the early 2000s has resulted in northward retraction of Arctic fish communities (e.g., bigeye sculpin and Greenland halibut) but expansion of the sub-Arctic and temperate communities (e.g., Atlantic cod and haddock) (26). The projected changes to fisheries estimated from our study under the 3.5°C warming scenario are very likely more severe than these observations.

Assumptions in four aspects of the models may affect our results: linearity between cumulative carbon emissions and ocean changes, uncertainty in projections of ecosystem drivers, evolutionary adaptation of marine species, and effects of ocean acidification. We show that the first assumption is generally valid for sea surface variables, but sea-bottom variables exhibit a lag to surface warming. Thus, our projected fisheries impacts may be more conservative (fig. S5). Second, accuracy of the projections is contingent on the outputs from the Earth system models (6, 11). Sensitivity analyses show that projected Δ MCP is most sensitive to temperature in all biomes, followed by NPP (fig. S7). At the same time, regional comparison of present-day ecosystem drivers with model projections shows that global and regional-scale patterns are relatively well represented for SST and surface O_2 , but not well simulated for bottom O_2 and NPP (fig. S8). Specifically, NPP is largely underestimated in all models in the large marine ecosystems (see supplementary materials). The uncertainties of projections of ecosystem drivers are particularly relevant to interpretation of our findings for small-scale and recreational fisheries that operate in inshore areas where these uncertainties are higher (7). Third, in DBEM, species' environmental preferences are based on the assumption that their current distributions are in equilibrium with the environment and species' traits do not evolve as environmental conditions change. Experimental studies suggest that there may be standing genetic variability that would allow species to adapt evolutionarily to warming, while transgenerational adaptations may also be possible for a few studied species (27). Species distribution models that assume adaptation potential for species' thermal tolerance project lower species local extinction, particularly in the tropics (18). However, the extent of such adaptation responses is unclear, as empirical analysis of species turnover in the tropics is currently understudied. Moreover, climate impacts on habitats such as coral reefs and estuaries may further increase the impacts on the associated species. Last, this study does not consider the effects of ocean acidification and potential interactions with other human drivers such as pollution or socioeconomic development on marine fisheries. Empirical evidence suggests that

exploited mollusks and echinoderms are sensitive to ocean acidification. Although the direct effects of ocean acidification on finfishes in general are inconclusive (28), indirect impacts on primary and secondary production, behavior, and trophic interactions may negatively affect finfish productivity (29). Thus, the addition of ocean acidification effects would further strengthen the conclusion that reduction in CO_2 emissions, which also reduces ocean acidification directly, reduces impacts on fisheries (5).

This study highlights the clear benefits for fisheries from achieving the Paris Agreement's target and fills in an important gap for the oceans on the implications of limiting global average temperatures to 1.5°C.

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SUPPLEMENTARY MATERIALS

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Author Contributions

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Tables S1 to S3

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PLANT SCIENCE

Precursor processing for plant peptide hormone maturation by subtilisin-like serine proteinases

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Peptide hormones that regulate plant growth and development are derived from larger precursor proteins by proteolytic processing. Our study addressed the role of subtilisin-like proteinases (SBTs) in this process. Using tissue-specific expression of proteinase inhibitors as a tool to overcome functional redundancy, we found that SBT activity was required for the maturation of IDA (INFLORESCENCE DEFICIENT IN ABSCISSION), a peptide signal for the abscission of floral organs in *Arabidopsis*. We identified three SBTs that process the IDA precursor in vitro, and this processing was shown to be required for the formation of mIDA (the mature and bioactive form of IDA) as the endogenous signaling peptide in vivo. Hence, SBTs act as prohormone convertases in plants, and several functionally redundant SBTs contribute to signal biogenesis.

Small posttranslationally modified peptides function as extracellular signaling molecules in plants (1). Such peptides control plant growth and development, as well as interactions between plants and their environment (1, 2). Addressing the biogenesis of plant peptide hormones, we focused on a peptide that

serves as a signal for the abscission of floral organs (petals, sepals, and stamens).

Abscission is on full display during autumn, when deciduous trees shed their leaves. The abscission process is also agriculturally important, as it facilitates the dispersal of fruits and seeds. In most flowering plants, including *Arabidopsis*

thaliana, floral organs abscise when pollination is complete (3, 4). The *IDA* (*INFLORESCENCE DEFICIENT IN ABSCISSION*) gene controls floral organ abscission in *Arabidopsis* (5). It codes for a secreted precursor protein that requires processing to release the biologically active IDA peptide (6). The peptide binds to the extracellular leucine-rich repeat domain of the receptor-like kinases HAE (HAESA) (7, 8) and HSL2 (HAESA-LIKE2) (6, 9) and promotes association with co-receptors of the SERK (SOMATIC EMBRYOGENESIS RECEPTOR KINASE) family (8, 10). Intracellular signaling then activates genes that execute the abscission process (9, 11). Floral organs are shed when polygalacturonases and other hydrolytic

enzymes degrade the pectin matrix of the middle lamella, resulting in cell separation in the abscission zone (11–13). Here we identify the proteases required for IDA precursor (proIDA) processing and peptide maturation. We show that proIDA is processed by subtilisin-like proteases (subtilases, or SBTs) and that several functionally redundant SBTs contribute to IDA maturation in vivo.

Among the 56 SBTs in *Arabidopsis*, 54 are predicted to be located in the cell wall, where they appear to serve redundant functions, as indicated by the lack of obvious phenotypes for most of the single-gene loss-of-function mutants (14, 15). The majority of *SBT* genes are expressed in floral receptacles (figs. S1 and S2), where sepals, petals, and stamens attach to the plant body. With such a large number of SBTs potentially involved in the abscission process, existing methods aiming to impair gene function have poor prospects of success. We therefore developed a biochemical approach, addressing functional redundancy at the level of enzyme activity, by tissue-specific expression of a protease inhibitor. Two proteins from *Phytophthora infestans* [EPI1 (extracellular proteinase inhibitor 1) and EPI10] were chosen, comprising atypical Kazal-domains as specific inhibitors of SBTs but of no other serine proteases tested (16, 17) (figs. S3 and S4). The inhibitor sequences were codon-optimized for expression in plants and equipped with a plant signal peptide for efficient secretion. Transgenic plants expressing EPI1a (the atypical Kazal-domain of EPI1) or EPI10 in abscission zones under control of the *IDA* promoter retained their flower organs and thus phenocopied the abscission defect of the *ida* mutant (Fig. 1 and fig. S5). Abscission was restored when these plants were treated with a 14-amino acid (aa) peptide (mIDA) comprising the conserved PIPP motif that is required for receptor activation (8) (Fig. 2A). We conclude that SBT activity is required for abscission and, furthermore, that at least some SBTs act upstream of signal formation. Consistent with a role in IDA signaling, the expression of *PGAZAT*, a polygalacturonase gene controlled by the IDA signaling pathway and required for cell separation in abscission zones (12, 13), was impaired in EPI10-expressing transgenics and restored to wild-type (WT) levels after treatment with mIDA, whereas

a longer peptide that still depends on processing for activation (eIDA) was inactive (Fig. 2, B and C).

To identify the precursor processing proteases, we selected 10 abscission-zone SBTs representing different SBT subfamilies and tested them for their ability to cleave the IDA precursor when coexpressed in *Nicotiana benthamiana* (fig. S6). Of the four proteases that affected IDA processing in this system, SBT4.12, SBT4.13, and SBT5.2 were partially purified from cell wall extracts of agro-infiltrated *N. benthamiana* plants and tested with a recombinant glutathione *S*-transferase (GST)–IDA fusion protein as the substrate. GST-IDA was processed by all three proteases and, in line with the abscission defect of the EPI1a- or EPI10-expressing transgenic plants, processing was sensitive to EPI10 inhibition (Fig. 3A). Low nanomolar concentrations of EPI1a and EPI10 were sufficient for half-maximal inhibition of SBT4.12, SBT4.13, and SBT5.2 (figs. S7 to S9). The dissociation constant (K_d) for the interaction of SBT4.13 with EPI1a was 23 ± 2 nM, whereas two binding events with $K_d = 13 \pm 2$ nM and 0.8 ± 0.1 μ M, respectively, were observed for the three-headed EPI10 inhibitor (Fig. 3B). The cumulative data—including (i) cleavage of the IDA precursor, (ii) coexpression with *IDA* in abscission zones, (iii) inhibition by the EPI inhibitors with inhibition and binding constants in the low nanomolar range, and (iv) the abscission defect of the EPI1a- or EPI10-expressing plants—support a role for SBT4.12, SBT4.13, and SBT5.2 in IDA processing and signal maturation.

As a candidate for the mature signal, each of the proteases released a 14-aa peptide from GST-IDA that was not formed in controls or when SBT activity was inhibited by EPI10 (fig. S10, A to C). The 14-aa peptide corresponds to part of the extended PIPP (ePIPP) motif (Fig. 4A) that is conserved between IDA and IDA-like proteins and contains the bioactive part of the protein (6). Within this region, a 12-aa peptide (PIPP) is minimally required for receptor (HAE) binding and co-receptor (SERK1) complex formation (8). The C-terminal Asn of PIPP is essential for receptor (HSL2) activation (18), and its carboxyl group needs to be free for binding (8). This Asn residue thus marks the C-terminal end of the endogenous IDA signal. Its

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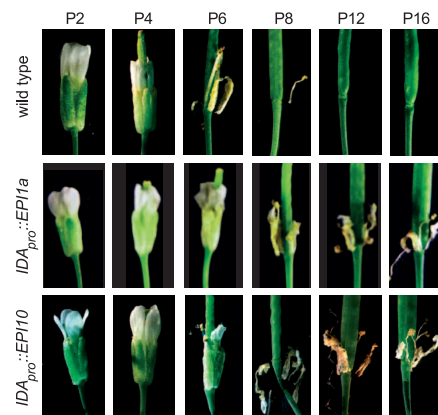


Fig. 1. The shedding of floral organs depends on subtilisin-like proteinase (SBT) activity in abscission zones. *Arabidopsis* Col-0 (WT) flowers complete abscission when they reach position P8 (the youngest flower after anthesis is in P1, older flowers are numbered consecutively). Transgenic plants expressing EPI1a or EPI10 under control of the *IDA* promoter retain their flower organs until fruit maturation.

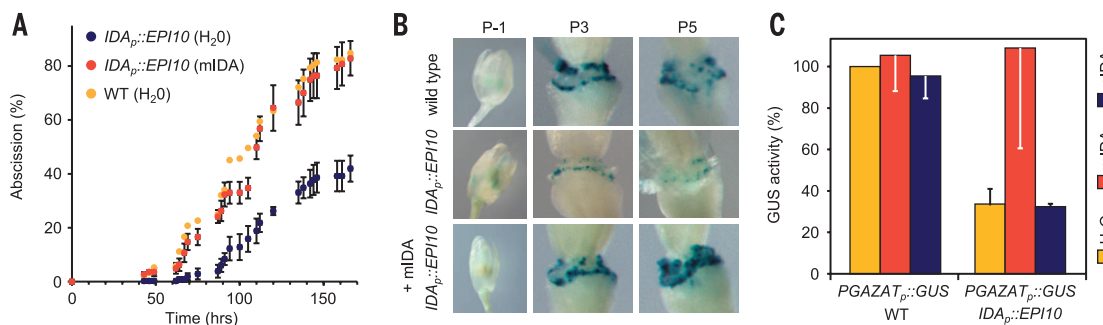


Fig. 2. mIDA restores abscission and IDA target gene expression in IDA_{pro}::EPI10 transgenic lines. (A) The delay of abscission (shown as percentage of flowers that shed their floral organs) observed in excised IDA_{pro}::EPI10 compared with WT inflorescences is overcome by mIDA (10 μ M, fed through the cut stem;

peptide sequence in Fig. 4A). Data represent the mean $\pm$ SD (error bars) for three independent transgenic lines, each analyzed in five experiments. (B) Reduced activity of the *PGATAT* promoter in receptacles of IDA_{pro}::EPI10 flowers is restored to WT levels by treatment with mIDA. (C) Quantification of GUS activity (given as percentage of the WT control), confirming the histochemical results shown in (B). Peptides (mIDA and eIDA) were supplied at 10 μ M. Data represent the mean of four experiments $\pm$ SEM (error bars).

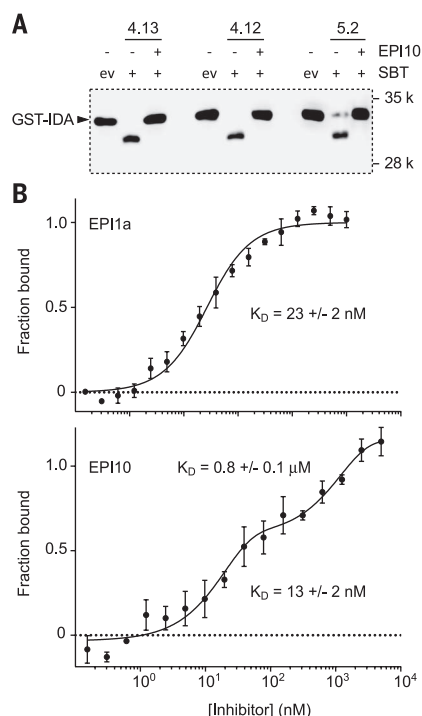


Fig. 3. Processing of the IDA precursor by SBTs is inhibited by extracellular proteinase inhibitors (EPIs). (A) Anti-GST immunoblot showing processing of recombinant GST-IDA upon addition of SBT4.12, SBT4.13, and SBT5.2 extracted from agro-infiltrated *N. benthamiana* plants (ev, empty vector control). Processing is inhibited in presence of EPI10. (B) Dissociation constants of SBT4.13-EPI inhibitor complexes determined by microscale thermophoresis. A biphasic saturation curve and two K_D values are observed for the three-headed EPI10 inhibitor (three experiments with independent preparations of the inhibitors). Error bars indicate SD.

N terminus is less clear, because N-terminal extension of PIPP by one or four amino acids does not alter receptor binding affinity (8, 18). We treated EPI10-expressing plants with the different peptides (Fig. 4A) to compare their abscission-inducing activity (Fig. 4B). In this bioassay, the 14-aa cleavage product was at least 10 times more active on a molar basis than PIPP or an N-terminally extended peptide (eIDA) (Fig. 4B). The residual activity of eIDA was lost when the peptide bond marking the N terminus of the 14-aa peptide (the Lys/Gly bond) was protected against proteolysis by N-methylation of the peptide backbone at this position (NMeIDA in Fig. 4B) (19). The highest abscission-inducing activity and the apparent requirement for processing of the Lys/Gly bond support the 14-aa peptide as the mature and bioactive form of IDA (mIDA).

In a genetic complementation experiment, we addressed the relevance of SBT-mediated precursor processing and mIDA formation for abscission in vivo. In contrast to WT *IDA* that is expected to restore abscission when expressed in the *ida* background, any precursor mutant that cannot be processed to produce bioactive mIDA will fail to do so. Failure of SBT-resistant

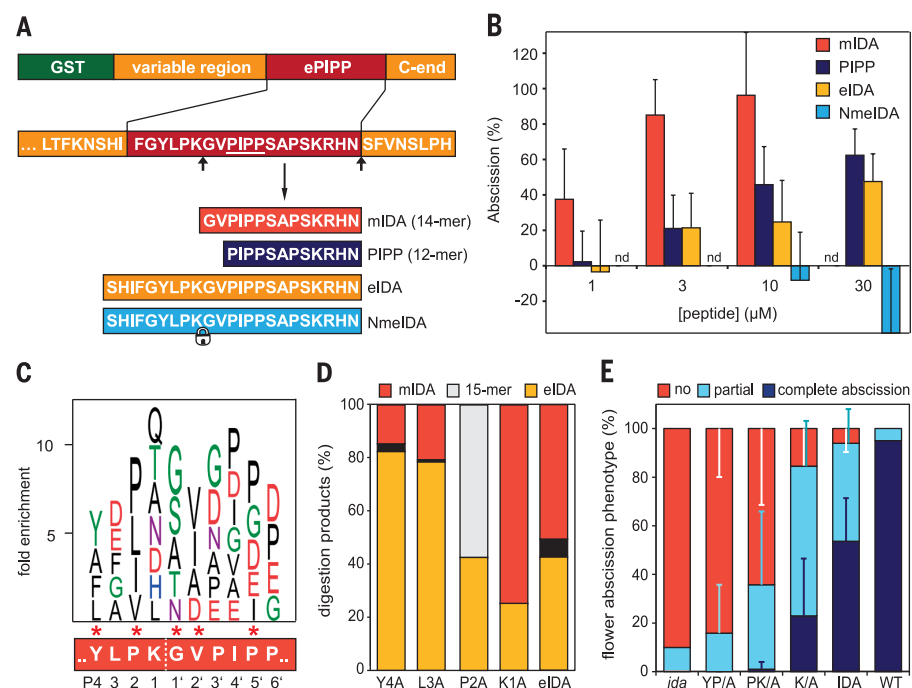


Fig. 4. Identification of mIDA and processing-site confirmation in vivo. (A) Representation of GST-IDA, processing sites, mIDA, and related peptides (not to scale). The lock icon marks the peptide bond protected against proteolysis by N-methylation. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr. (B) Dose-dependent abscission-inducing activity of the peptides is shown relative to water-treated *IDA_{pro}*:EPI10 and WT plants set to 0 and 100%, respectively [mean $\pm$ SD (error bars) for $n = 6$ biological replicates, including three independent transgenic lines]. (C) Sequence logo showing residues preferred by SBT4.13 upstream (P1 to P4) and downstream (P1' to P6') of the cleavage site (K/G), as analyzed by PICS (Proteomic Identification of Cleavage Sites). Asterisks highlight positions in which the residue that was most highly enriched over natural abundance matches the IDA precursor sequence. (D) Using Ala-substituted eIDA analogs as substrates of SBT4.13, P2-Pro and P4-Tyr were confirmed as the two most important residues for mIDA formation. Bars show the abundance of substrate (yellow) and products (red and gray; black represents the sum of all other peptides) in the reaction mixture as the percentage of all peptides identified by mass spectrometry. When P2-Pro was substituted (P2A), a miscleaved 15-aa peptide, rather than mIDA, was produced. (E) Genetic complementation of the *ida* abscission defect by *IDA_{pro}*:*IDA*-GFP and Ala-substituted mutants. Partial abscission refers to plants that retained flower organs in some positions older than P8 [mean $\pm$ SD (error bars) of 6 to 10 independent transformants].

mutants of the IDA precursor to complement the abscission defect of *ida* would thus confirm SBT-dependent processing as a prerequisite for IDA maturation. To generate IDA variants that are resistant to SBT cleavage, it is essential to know how these proteases recognize their substrates. In a proteomics approach [Proteomic Identification of Cleavage Sites (PICS)] (20), substrate libraries comprising more than 10,000 peptides were digested with SBT4.13 purified to homogeneity from agro-infiltrated *N. benthamiana* plants (fig. S11), and cleavage sites were identified by mass spectrometry. SBT4.13 was found to tolerate several different amino acids on both sides of the scissile bond (Fig. 4C); therefore, our first impression was that the protease is rather promiscuous with respect to cleavage site selection. However, with exception of the P1 position (the one immediately upstream of the cleavage site), the residues that were most highly preferred by SBT4.13 around the cleavage site of its peptide substrates were found in the corresponding posi-

tions flanking the processing site of the IDA precursor. Thus, substrate selectivity of SBT4.13 is not determined by a single amino acid in P1, but rather by an extended sequence motif including residues on both sides of the scissile bond. Pro and Tyr in P2 and P4, as well as Gly, Val, and Pro in P1', P2', and P5', seemed particularly important (Fig. 4C). Using alanine-substituted synthetic peptides as substrates for SBT4.13, P2-Pro and P4-Tyr were confirmed as the two most important residues for substrate recognition on the nonprime side of the processing site (Fig. 4D). These substitutions are not part of mIDA and should thus not affect the bioactivity of the processing product.

To test whether recognition by subtilases and cleavage of the Lys/Gly bond are required for the generation of mIDA as the abscission signal in vivo, site-directed *IDA* mutants with Ala substitutions in P1, P2, and/or P4 were introduced into the *ida* background. Expression of the precursor variants in abscission zones was confirmed by the use of a C-terminally fused green fluorescent

protein (GFP) tag (fig. S12). Consistent with the irrelevance of P1-Lys for cleavage site recognition (Fig. 4, C and D), the P1-Lys/Ala mutant (K/A in Fig. 4E) complemented the abscission defect of *ida* to almost the same extent as WT IDA-GFP. Substitution of Pro (P) in P2 in addition to P1-Lys (PK/A) reduced the ability to complement the mutant phenotype, and the P2-P4 double mutant (YP/A; Y, Tyr) was essentially inactive (Fig. 4E). Similarly, an ePIPP expression construct genetically complemented the *ida* mutant, and activity was impaired when Pro in P2 was substituted by Ala (P/A) (fig. S13).

Failure of the SBT-resistant precursor variants to restore abscission in the *ida* mutant confirms the IDA precursor as a physiological substrate of SBTs and indicates a role for SBTs in signal maturation. We conclude that precursor processing at the Lys/Gly bond is a prerequisite for the biogenesis of the abscission signal in *Arabidopsis* flowers, implicating the 14-aa peptide mIDA as the endogenous signal. Previous studies favored the 12-aa PIPP peptide (Fig. 4A) as the abscission signal (8, 18). We cannot exclude further trimming of the N terminus and the generation of PIPP by an unidentified aminopeptidase after initial cleavage of the Lys/Gly bond. However, considering the reduced activity of PIPP as compared with mIDA (Fig. 4B), this would decrease rather than increase signal intensity.

The close correlation observed between the amino acid residues required for the biogenesis of mIDA *in vivo* and for substrate recognition by SBT4.13 *in vitro* implicates SBT4.13 in the maturation process. However, abscission is normal in SBT4.13 single-gene loss-of-function mutants (fig. S5); therefore, SBT4.13 cannot be the only protease responsible. SBT4.12 and SBT5.2 are also involved, as they are coexpressed with IDA and SBT4.13 in abscission zones (fig. S2) and show the same cleavage specificity with respect to proIDA processing (fig. S10). The activity of several redundant subtilases is thus responsible for the biogenesis of the abscission signal.

Although AtSBT6.1, which is one of two intracellular SBTs and the ortholog of human site-1 protease, has previously been implicated in precursor processing (15, 21–24), peptide hormone maturation by cognate SBTs was hitherto obscured by functional redundancy in the large SBT family. Using EPI inhibitors as a tool to overcome this functional redundancy, we demonstrated a role for SBTs in IDA maturation and abscission. Tissue-specific expression of EPI inhibitors will be instrumental for further analysis of SBTs in peptide hormone biogenesis. Tissue-specific expression of enzyme inhibitors may also be useful in other systems for the analysis of functionally redundant enzymes.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6319/1594/suppl/DC1
Materials and Methods
Figs. S1 to S14
Table S1
Reference (25)

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STRUCTURAL BIOLOGY

Crystal structure of unlinked NS2B-NS3 protease from Zika virus

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Zika virus (ZIKV) has rapidly emerged as a global public health concern. Viral NS2B-NS3 protease processes viral polypeptide and is essential for the virus replication, making it an attractive antiviral drug target. We report crystal structures at 1.58-angstrom resolution of the unlinked NS2B-NS3 protease from ZIKV as free enzyme and bound to a peptide reversely oriented at the active site. The unlinked NS2B-NS3 protease adopts a closed conformation in which NS2B engages NS3 to form an empty substrate-binding site. A second protease in the same crystal binds to the residues K14K15G16E17 from the neighboring NS3 in reverse orientation, resisting proteolysis. These features of ZIKV NS2B-NS3 protease may accelerate the discovery of structure-based antiviral drugs against ZIKV and related pathogenic flaviviruses.

Zika virus (ZIKV) has spread across the world rapidly and is becoming a serious public health concern owing to its link to severe neurological diseases such as fetal microcephaly and Guillain-Barré syndrome in adults (1, 2). Specific antiviral therapeutics against ZIKV are urgently needed to fight this pandemic. ZIKV belongs to the Flaviviridae family, *Flavivirus* genus, which contains important human pathogens including dengue virus (DENV), West Nile virus (WNV), yellow fever virus, Japanese encephalitis virus, and tick-borne encephalitis virus (3–5).

The genomes of these viruses encode a polyprotein that is processed into three structural proteins (capsid, membrane, and envelope proteins) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by both the host proteases and the viral NS2B-NS3 protease. As such, the NS2B-NS3 protease is an attractive target for antiviral drug development (6, 7). NS3 contains a trypsin-like fold and carries the conserved catalytic triad (H51, D75, and S135). The small transmembrane protein NS2B anchors NS3 to the endoplasmic reticulum membrane, and together they form an active enzyme for substrate recognition and catalysis (7–10). The minimal cofactor region of the NS2B comprises the hydrophilic residues 49 to 97 (11). The N-terminal 18 residues (49 to 67) of NS2B cofactor support the proper fold of NS3 protease by forming a beta strand inserted into the protease domain (12, 13). The C-terminal part of NS2B cofactor (residues 68 to 96) forms a beta-hairpin to create the S2 and S3 pockets in the substrate-binding site of NS3 protease (13–15).

Crystal structures of the proteases from DENV (13, 14, 16), WNV (13, 15, 17, 18), Murray Valley

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encephalitis virus (19), and ZIKV (20) have been determined using the same construct of NS2B-NS3 connected with an artificial glycine-rich linker. In the absence of an inhibitor, the C-terminal part of NS2B cofactor is flexible and often disordered (open conformation) (13, 16, 18, 19). Inhibitor-bound structures adopt a compact complex where the NS2B fragment wraps around the NS3 protease and makes direct contacts with the inhibitors (closed conformation) (13–15, 17–20). Although solution nuclear magnetic resonance (NMR) studies of DENV and WNV proteases have suggested that the free enzyme is able to form the closed conformation in solution (21–23), it is still unclear whether the closed conformation is stabilized by the binding of a ligand. For ZIKV, we have discovered that the artificial linker introduces steric hindrance and alters the substrate (inhibitor)-binding behavior, suggesting that the unlinked binary ZIKV NS2B-NS3 protease (bZiPro) is preferable for studying the enzyme behavior and for inhibitor development (24). In this regard, it is important to characterize the dynamic behavior of ZIKV NS2B-NS3 protease in detail.

Here we report the crystal structure of bZiPro at a resolution of 1.58 Å (Fig. 1 and table S1). The refined model consists of four bZiPro molecules in one unit cell labeled according to the peptide chain IDs AB, CD, EF, and GH (fig. S1). There are three molecules of free enzyme and one bound to the K14K15G16E17 tetrapeptide from the neighboring NS3 N terminus in an unusual reverse orientation (Fig. 1 and fig. S1). NS2B cofactor is in the closed conformation—both the N- and C-terminal regions are folded into a β -sheet conformation—and in a complex with the NS3 protease domain in all four bZiPro molecules. The structures of bZiPro free enzyme are virtually identical to the peptide-bound bZiPro (AB) with root mean square deviations (RMSDs) of 0.25 to 0.34 Å after superimposition (Fig. 1D, fig. S1B, and table S2). In addition, the temperature factor of the individual NS2B chain is also comparable to that of the partner NS3, indicating that the NS2B and NS3 form a stable complex in the crystal (fig. S1C). Furthermore, bZiPro is very similar to eZiPro [ZIKV NS2B-NS3 protease after self-cleavage; Protein Data Bank (PDB) code, 5GJ4], which has a RMSD of 0.45 Å for 162 C α atoms, and gZiPro (the single chain ZIKV NS2B_{49–96}-G4SG4-NS3Pro for NS2B-NS3 protease with a glycine-rich linker; PDB code, 5LC0), which has a RMSD of 0.52 Å for 167 C α atoms (Fig. 1, fig. S1A, and table S2) (20, 24). The unlinked ZIKV NS2B-NS3 protease appears to contain a preformed stable substrate-binding pocket that does not undergo further substantial conformational changes upon substrate or inhibitor binding.

Unexpectedly, one bZiPro molecule (AB) binds to the N-terminal K14K15G16E17 tetrapeptide sequence extended from the neighboring NS3 protease (H' chain) (Fig. 1A and fig. S1A). We identified the residues E12 to T18 from the neighboring NS3 N-terminal region in the electron density map and built them into the structure unambiguously (Fig. 1F). The same residues are

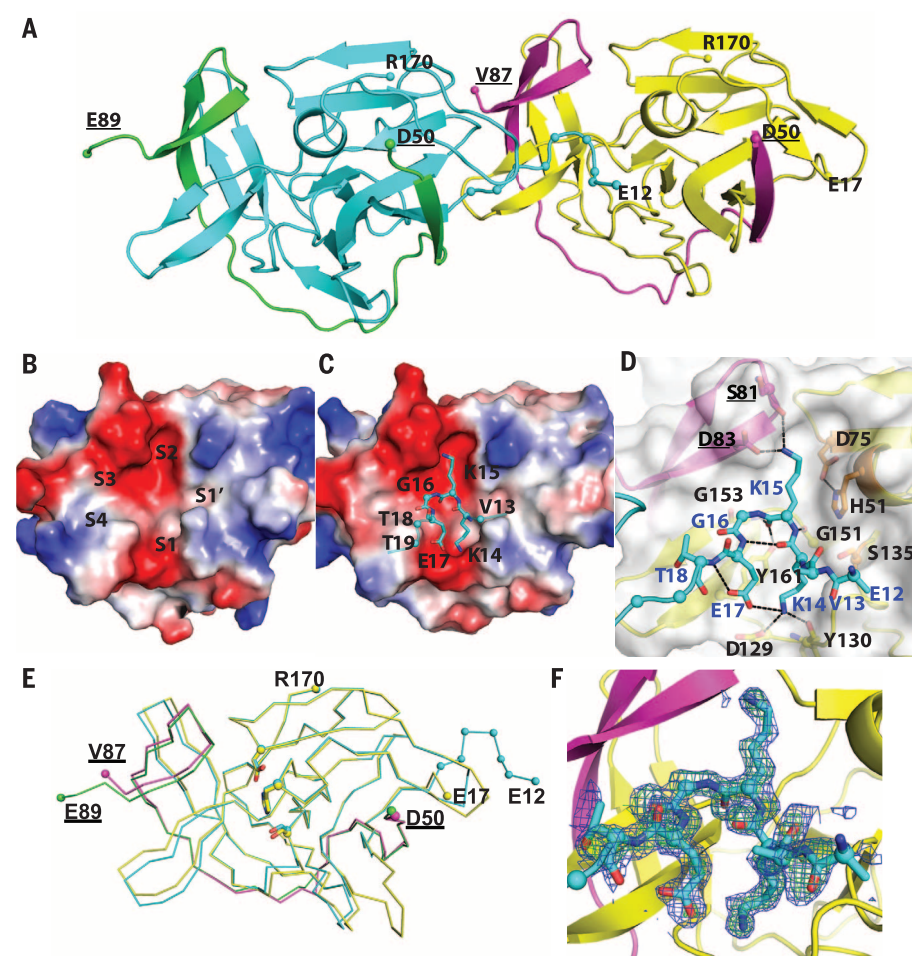


Fig. 1. Crystal structure of bZiPro. (A) The free enzyme and peptide-bound ZIKV protease structures determined from the bZiPro crystal. In the free enzyme form, NS2B is colored in green and NS3 in cyan. In the peptide-bound form, NS2B is colored in magenta and NS3 in yellow. The N- and C-terminal residues of NS2B and NS3 are labeled. (B) Electrostatic view of bZiPro as free enzyme with an empty preformed substrate-binding pocket (pockets are labeled). (C) Electrostatic view of bZiPro in complex with the NS3 N-terminal peptide K14K15G16E17 in an unusual reversed orientation. Electrostatic surfaces were colored using PyMOL by electrostatic potential at neutral pH from -5 kT (red) to $+5$ kT (blue). (D) Close-up views of the interactions between N-terminal residues K14 to E17 from a neighboring NS3 (in cyan) and the residues from bZiPro. (E) Superimposition of the two structures of bZiPro, shown as ribbons. The RMSD is 0.34 Å for 152 C α atoms. (F) A simulated annealing omit $mF_o - DF_c$ map of the KKGE reverse peptide within the bZiPro crystal structure is contoured at 3σ in green mesh. A $2mF_o - DF_c$ electron density map is contoured at 1σ in blue. Throughout, residues from NS2B are underlined. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

disordered in the remaining three bZiPro, eZiPro, and gZiPro structures, suggesting that the AB-H' protease complex might be a crystallographic artifact (20, 24). Nonetheless, the structure does capture the protease in complex with a reverse peptide. The tetrapeptide K14K15G16E17 folds into a small hairpin loop to occupy the active site. Specifically, the K14 ϵ -amino group occupies the S1 pocket and forms hydrogen bonds with D129 and Y130. Residue K15 contributes the most to the overall binding: Its side chain ϵ -amino group forms hydrogen bonds with S81 and D83 in the S2 pocket, and its main chain forms hydrogen bonds with G151 and G153 of NS3 protease. G16 does not form any interactions with the pro-

tease and leaves the S3 pocket completely empty, similar to NS2B G129 in the eZiPro structure. The E17 side chain stacks with the aromatic ring of Y161 of the protease and partially occupies the S1 pocket with K14. Notably, the hairpin is partially stabilized by the intramolecular hydrogen bonds: one between the backbone carbonyl oxygen atom of K14 and the amide nitrogen atom of E17, and two between the side chain carboxylic group of E17 and the ϵ -amino group of K14 and backbone amide of T18 (Fig. 1D). The specific conformation of the reverse peptide does not allow the catalytic residue S135 to establish the hydrogen-bonding relay and form the reactive oxyanion species. It also prevents the carbonyl

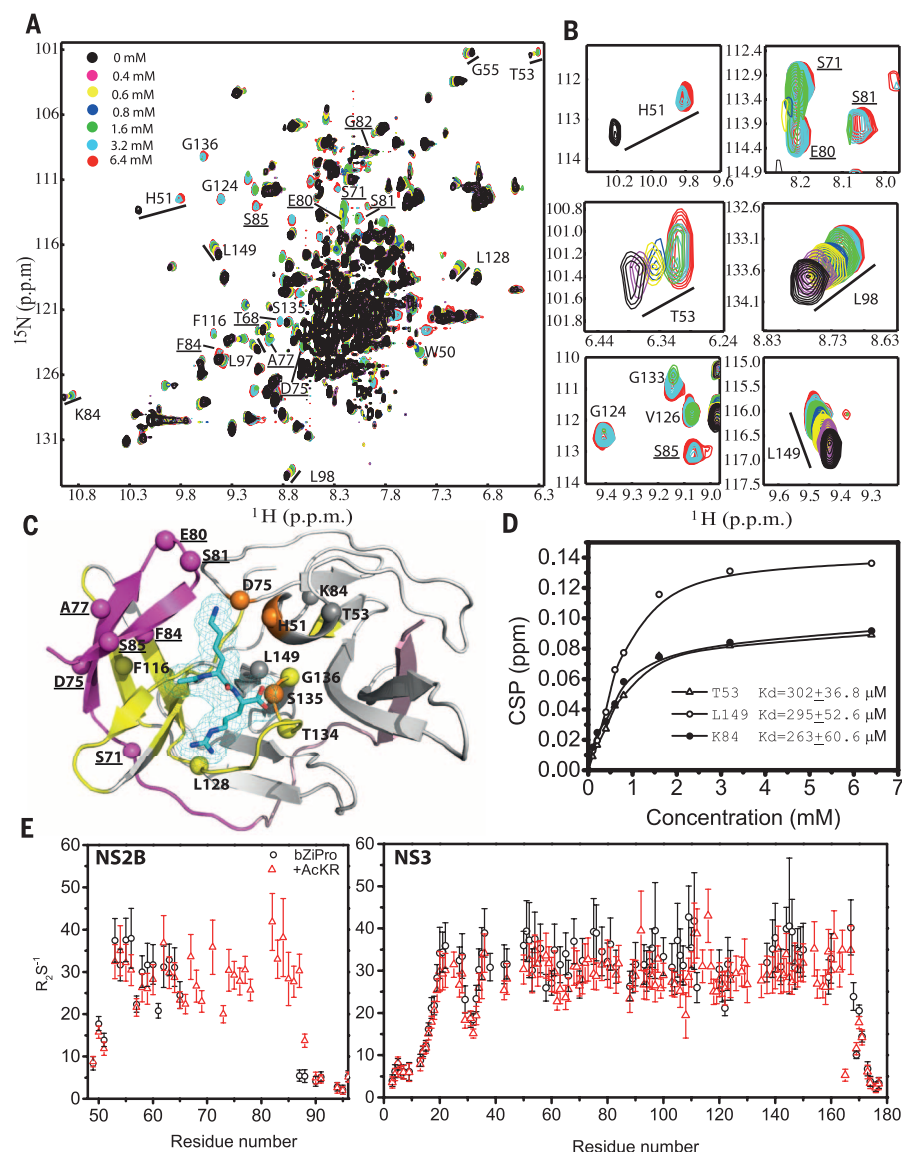


Fig. 2. Structural dynamics of bZiPro in solution. (A) Overlay of ^1H - ^{15}N HSQC spectra of 0.8 mM bZiPro in the absence (black) and presence (colors) of different amounts of AcKR peptide. Some residues that exhibited line broadening or chemical shift changes are labeled. (B) Enlarged view of several residues that exhibited cross peaks and chemical shift perturbation upon AcKR binding. (C) Affected residues shown in (A) and (B) are highlighted as spheres and labeled on the structure of protease. The structure of AcKR is modeled in the active site on the basis of the structure of eZiPro. Residues colored in yellow (magenta) from NS3 (NS2B) could not be unambiguously assigned because of line broadening and miss of signal connection in the free bZiPro enzyme. In (A) and (C), residues from NS2B are underlined. (D) Residues T53, K84, and L149, which exhibited an averaged chemical shift change of more than 0.08 parts per million (ppm), are used for affinity estimation. Overall, AcKR binds to bZiPro very weakly. (E) Protein flexibility analysis. The ^{15}N R_2 values of 0.8 mM bZiPro in the absence (black) and presence (red) of 3.2 mM AcKR peptide are plotted against residue number. Error bars were obtained from NMRView (27).

group of the peptide to be positioned close to S135. This reverse peptide could not be cleaved by the protease, and the complex structure may guide structure-based inhibitor design (25).

To reveal the structural dynamics of bZiPro in solution, we carried out NMR studies. The acquired ^1H - ^{15}N heteronuclear single-quantum coherence (HSQC) spectrum of bZiPro exhibited

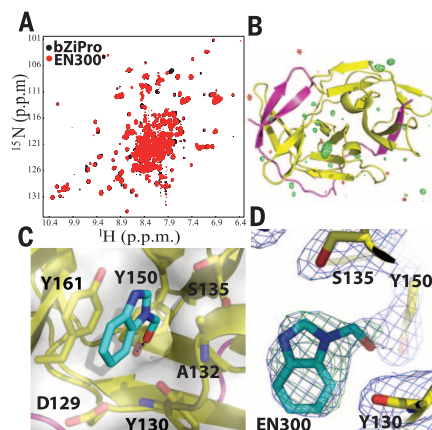
dispersed cross peaks, indicating that bZiPro in solution is a well-folded structure with similar secondary structures to those of the crystal structure (Fig. 2A and fig. S5). As a result of signal broadening in the ^1H - ^{15}N HSQC spectrum of bZiPro, there were missing peaks for residues 47 to 49, 114 to 119, 123 to 133, 153 to 158, and 161 to 166 of NS3 (Fig. 2, B and C, and fig. S4B).

To determine whether the protease exists in the closed conformation in solution with an open active site for inhibitor binding, we titrated bZiPro with acetyl-lysine-arginine (AcKR) (Fig. 2), a dipeptide known to weakly inhibit WNV protease activity (median inhibitory concentration ≥ 100 μM) (26). The ^1H - ^{15}N HSQC spectra of bZiPro in complex with AcKR were better resolved than those of bZiPro alone, and cross peaks of many residues from both NS2B and NS3 reappeared (Fig. 2 and fig. S4). The ^1H - ^{15}N HSQC profile of bZiPro-AcKR is very similar to that of eZiPro, implying that the cleavage product peptides can bind to the protease active site similarly, in cis or trans configuration (fig. S6). As evident in the ^1H - ^{15}N HSQC profile, residues from the catalytic triad and their surroundings undergo local environmental changes upon peptide binding (fig. S7). H51 first exhibited line broadening in the presence of 0.4 to 1.6 mM AcKR peptide. Its peak reappeared at a different position when an excess amount of inhibitor (3.2 to 6.4 mM) was used to saturate the active site. Cross peaks of NS3 residues—such as 114 to 119, 125 to 130, 133 to 134, 153 to 158, 161, and 163 to 166—and the C terminal region of NS2B cofactor also appeared as a β -hairpin (Fig. 2 and fig. S5). We also observed a second population of narrowly dispersed cross peaks of unstructured NS2B in the spectra of both bZiPro and the bZiPro-AcKR complex. These peaks were identical to those of the free NS2B cofactor and were unresponsive to AcKR titration (figs. S4 and S5). There seemed to be free NS2B cofactor after dissociation from NS3 in the bZiPro protein samples.

The overall flexibility changes of bZiPro upon binding to AcKR were further probed by ^{15}N R_1 , R_2 , and heteronuclear nuclear Overhauser effect (hetNOE) experiments (Fig. 2E and fig. S8). Consistent with previous structural studies, the N-terminal part of NS2B cofactor exhibited very similar dynamic patterns to those of NS3, independent of AcKR binding; this segment of NS2B forms a stable complex with NS3. The C-terminal β -hairpin region also forms a stable structure in solution, evidenced by the high hetNOE values. Overall, bZiPro appears to have a very dynamic local environment at both the active site and the interface between the C-terminal part of NS2B cofactor and NS3, which is stabilized by the addition of a peptide inhibitor occupying the S1 and S2 pockets (Fig. 2). The free bZiPro adopts a closed conformation in solution, and the line broadening observed for the residues may be due to the presence of minor local conformational exchanges of the C terminus of NS2B and residues close to the active site of NS3. Similarly, it has been reported that minor populations or local conformational exchanges can lead to the disappearance of cross peaks for WNV protease (22).

To prove that the bZiPro construct is suitable for antiviral drug design, we determined the crystal structure of bZiPro in complex with a low-molecular-weight compound, EN300 [chemical name, (1H-benzo[d]imidazol-1-yl)methanol] (Fig. 3). The compound was identified through a

Fig. 3. Structure of bZiPro in complex with a compound fragment. (A) Overlay of ^1H - ^{15}N HSQC spectra of bZiPro in the absence (black) and presence (red) of the compound EN300. (B) The simulated annealing omit map of the bZiPro crystal structure is contoured at 4σ in green mesh. (C) Close-up view of the interactions between NS3 and the compound. (D) Electron density map at the compound binding site. The $2mF_o - DF_c$ electron density map is contoured at 1σ in blue, and the simulated annealing omit map is contoured at 3σ in green. Clear electron density is observed for the benzimidazole portion of EN300, forming a π -stacking interaction with Y161. Weaker electron density observed around the hydroxyl group could be indicative of partial hydrolysis of the compound.



fragment screen and was found to be able to stabilize bZiPro in a thermal shift assay (fig. S9). Changes in the ^1H - ^{15}N HSQC spectra upon binding not only confirmed the direct binding of the compound to bZiPro, but also showed that it did not cause any large-scale conformational changes to the protein (Fig. 3A). Indeed, EN300 is sandwiched between the Y161 aromatic ring and A132 by stacking interactions and forms a hydrogen bond with Y150 (Fig. 3). It only partially occupies the S1 pocket and forms no direct contact with NS2B cofactor. Therefore, the closed conformation of bZiPro is captured again in this structure, which is unlikely to be attributable to the binding of the small compound. The protein-ligand complex structure serves as a starting point to guide further chemical modifications for optimizations in binding potency and inhibition of protease activity inhibition in a targeted manner (movie S1).

We show that the crystal structure of free unlinked ZIKV NS2B-NS3 protease exists in the closed conformation. Solution NMR studies confirmed that free protease is predominantly in a closed conformation, with local conformational dynamics occurring at the NS2B-NS3 binding interface. Substrate peptide binding does not induce further substantial conformational changes. This finding has relevance for the discovery of antiviral drugs targeting ZIKV protease. The high-resolution crystal form that we present will

be of practical use because these crystals can be readily soaked or cocrystallized with inhibitors. Chemical library screens including fragment-based screening will have a higher likelihood of obtaining potent lead compounds and achieving successful structure-based lead optimization. We also report the cocrystal structure of a flaviviral protease bound to a peptide in a C-to-N orientation. The reverse direction of the peptide bond is unable to form a tetrahedral intermediate at the protease active site and is therefore not cleavable. Although this might be a crystal artifact, the good fit of the peptide within the protease pocket provides an attractive starting point for developing novel peptidomimetic inhibitors, such as cyclic peptides.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6319/1597/suppl/DC1
Materials and Methods
Figs. S1 to S9
Tables S1 and S2
References (28–43)
Movie S1

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"Diverse and ambitious environment at SciLifeLab"

SciLifeLab, Science for Life Laboratory, is a Swedish research center within molecular biosciences with focus on health and environment. To further strengthen the research environment at SciLifeLab the center regularly recruits young, talented research leaders to become SciLifeLab fellows. Each fellow is recruited by one of the center host universities and receives funding from them.

One of the SciLifeLab fellows is Jens Carlsson, who was recruited by Uppsala University/SciLifeLab from Stockholm University, Sweden, in 2015. After finishing his postdoctoral research at the University of California in San Francisco, Jens Carlsson returned to Sweden where he was born.

"I received a start-up grant from a Swedish foundation that gave me the opportunity to build up an independent research group at Stockholm University. I wanted to return to Sweden because family life, with two small children, became very complicated in the US. In Sweden, we are spoiled with generous parental leave and

subsidized day care – life is easier here"

"I was working at the Stockholm node of SciLifeLab when I applied for the fellows position. It is a wonderful place that connected me to the other universities represented at the center in ways that had not happened before. The relatively young community at SciLifeLab creates a high ambition level and the international recruitment has given rise to a great and diverse environment, which I think will benefit research in Sweden for many years to come."



Jens Carlsson

Photo: Håkan Lindgren

SciLifeLab – a national resource

SciLifeLab is a Swedish research center within molecular biosciences with focus on health and environment. It is also a national center with the mission to develop, use and provide advanced technologies. The center infrastructure encompasses a multitude of biomolecular technologies and bioinformatics services. National funding makes SciLifeLab's services and expertise available to researchers in all of Sweden.

The center is a joint effort by four Swedish universities (Karolinska Institutet, KTH Royal Institute of Technology, Stockholm University and Uppsala University). Founded in 2010, the center today encompasses more than 1 200 researchers mainly located in and around the two center nodes in Stockholm and Uppsala.

Looking to recruit more people

Jens Carlsson's research focuses on the family of G-Protein Coupled Receptors (GPCRs). He uses computer models of these receptors to understand how they work at the atomic level. As GPCRs are important drug targets, Jens and his colleagues also design small molecules that modulate receptor activity.

"Around 30-40% of all drugs target GPCRs so more or less everyone will take one of these at some point in their life," Jens Carlsson said. "Instead of testing millions of molecules experimentally as they do in the pharmaceutical industry, we can screen them on a super computer center over night and identify the most promising candidates at essentially no cost."

Jens Carlsson's first PhD student recently graduated and today his group consists of five researchers. He is now looking to recruit more students.

"I just had an interview with a candidate who I hope will be the first in my group to do experimental work. This will be very exciting because it will give me the possibility to quickly test computational predictions experimentally and thereby explore many new ideas."

An abstract graphic featuring a grid background. In the upper left, a cluster of yellow circles of various sizes. In the upper right, a cluster of red circles. In the lower left, a cluster of blue circles. In the lower right, a cluster of green circles. In the center, two large, textured blue spheres resembling cells or RNA molecules. The larger sphere on the right is covered in white text representing RNA sequences, including 'GAUGCGGCT', 'UAAGAUCUUC', 'UACGCCGUAC', 'CUUACUGAA', 'CAGUCUUA', and 'GCUUACU'. The smaller sphere on the left also has some text, including 'UACUAG', 'UAGUUG', and 'UAGUUG'.

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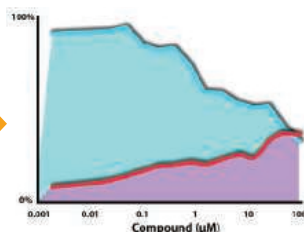
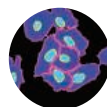
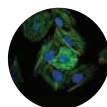
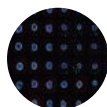
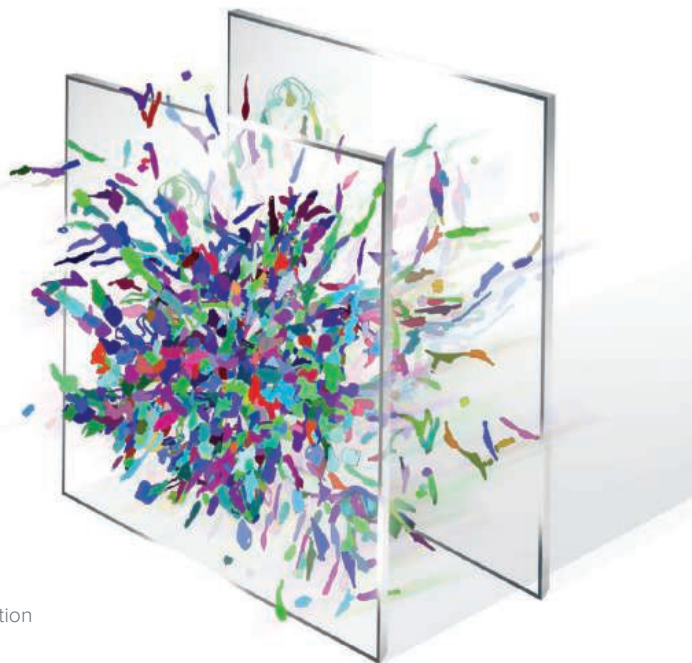
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this extra flexibility. Herolab UV analysis lamps service a multitude of laboratory operations, including fluorescent particle detection in biological and chemical applications, destruction-free material testing, fissure tests, adhesive curing, compound potting, and coating testing. Each product meets all current safety standards and will give many years of service.

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www.herolab.de/index.php/en

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Eppendorf expands its portfolio of rigid-wall, single-use vessels for fermentation. The Eppendorf BioBLU 3f was developed specifically for microbial bioprocessing in working volumes of 1.25 L to 3.75 L, extending the full range of operation for single-use fermentation from 60 mL to 3.75 L. These vessels specifically address the demands of high-cell-density fermentation of bacteria, yeasts, and fungi. Robust magnetic overhead drives featuring Rushton-type impellers support agitation rates up to 1,200 rpm and provide high-performance mass transfer. Eppendorf's exclusive integrated cooling baffles enable efficient heat removal for exothermic processes. The 3f's rigid-wall, industrial stirred-tank design ensures scalability and simplifies technology transfer. Its monolayer polymer material mitigates risk and uncertainty with regard to leachables and extractables, and its single-use bioreactors are designed as drop-in replacements for existing autoclavable fermentation vessels. They can be operated with Eppendorf bioprocess control stations BioFlo 115, BioFlo 310, and BioFlo 320.

Eppendorf

For info: 800-645-3050
www.eppendorf.com/BioBLUf

UV Analysis Lamps

Herolab produces a wide range of UV analysis lamps for short-, medium-, and long-wave UV, covering wavelengths of 254 nm, 312 nm, and 365 nm—thus there is a lamp for most laboratory applications. The lamps are constructed with powder-coated, lightweight metal housings. They have ergonomic handles and also offer a stand for hands-free operation. The range starts with small benchtop lamps and goes up to larger, industrial-size germicidal lamps. It is also possible to provide multiwave units for customers who need

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PROFESSOR AND CHAIR, Department of Genetics School of Medicine The University of North Carolina at Chapel Hill

The School of Medicine of the University of North Carolina at Chapel Hill (UNC-CH) seeks a well-qualified academic leader to serve as Chair of its Department of Genetics. Interested individuals should view the description and apply online at website: <http://unc.peopleadmin.com/postings/110733>.

UNC-CH is an Equal Opportunity/Affirmative Action Employer with a strong institutional commitment to excellence through diversity. All qualified applicants will receive consideration for employment without regard to gender, race, color, religion, sex, sexual orientation, national origin, disability, age or protected veteran status.

ALLEN INSTITUTE for BRAIN SCIENCE- ASSISTANT INVESTIGATOR

This Assistant Investigator will lead a team in developing a classification effort toward understanding the organization of cell types in the neocortex, building on and guiding the use of a set of in-house high-throughput quantitative single cell phenotyping pipelines applied to the mouse and human cortex. This multidisciplinary project requires knowledge of principal data types used for quantitative single-cell analysis in modern neuroscience, including single cell transcriptomics, electrophysiology, dendritic and axonal morphology and cell type-specific synaptic connectivity. This investigator will play a key role in generating a computational framework for discretizing the cell types of the cortex to create a cellular ontology, deriving probabilistic multimodal representations and testable hypotheses about the biological relevance of these types, & identifying conserved and divergent features between mouse and human.

QUALIFICATIONS: Ph.D. in computational neuroscience, biology, physics, engineering, or related computational discipline Nine or more years of post-PhD working experience in computational neuroscience with interest in the cell type problem Experience and technical knowledge of neuroscience, modeling, and machine learning paradigms, with supporting publication record Exceptional knowledge of quantitative clustering algorithms and/or ontological/phylogenetic tree building.

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POSTDOCTORAL FELLOWSHIP is available to pursue research supported by NIH grants. Studies will address the effects of plant products such as resveratrol, indoles and cannabinoids on inflammation, autoimmunity and cancer. Other projects include studies on the role of CD44, estrogens and dioxins on immune response. Ph.D. in Immunology is required. Send c.v. and 3 references to **Dr. Mitzi Nagarkatti, Carolina Distinguished Professor and Chair, Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC 29208** or e-mail: postdoc17@uscmed.sc.edu. USC Columbia is an EOAA Employer and encourages applications from women and minorities.

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MY WORK IS A STORY OF ACTION.

I get things moving, and that helps save lives. When a little boy needed us to deliver a customer's cutting-edge drug to help him fight a brain tumor, I did everything I could to keep the job moving forward. Thanks to the hard work of everyone on our team, we were able to deliver the drug three days ahead of the initially established "rush" date—meaning there was no delay in the boy's treatment.

In the BioPharma Services division, we make the pharmaceutical clinical trial supply chain simpler and safer. We know patients are waiting for medications, and we supply more than products—we supply hope. It's true that we don't always know the specific impact our job will have on our customers. But working for Thermo Fisher Scientific, I know that everything I do is playing a part in improving people's lives—and maybe even giving someone another chance.

When you're ready to move forward in your career, you'll discover that at Thermo Fisher Scientific, each one of our 50,000 extraordinary minds has a unique story to tell. And we all contribute to a singular mission—enabling our customers to make the world healthier, cleaner and safer.

Sarah

Project Manager

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DIRECTOR UNIVERSITY OF PITTSBURGH CANCER INSTITUTE

The University of Pittsburgh School of Medicine and UPMC (University of Pittsburgh Medical Center) seek an internationally recognized, visionary, high-energy leader to serve as director of the University of Pittsburgh Cancer Institute (UPCI; <https://upci.upmc.edu/>). The 2015 renewal of UPCI's substantial National Cancer Institute (NCI)-funded Comprehensive Cancer Center grant was ranked as "outstanding" and extends through 2020. The center's current research funding totals more than \$148 million (direct and indirect costs). UPCI serves Western Pennsylvania's population of more than 6 million and an expanding number of international locations. UPCI sees more than 25,000 new patients annually, with net patient revenue of more than \$724 million in FY 2015. The institute's 337 faculty, representing more than 43 disciplines, conduct research that reflects a comprehensive approach to understanding the causes of cancer, from the molecular to the environmental levels, as well as preventing and treating the many types of malignant diseases. In the last five years, UPCI has recruited more than 50 laboratory and clinical investigators from leading centers globally and is the single largest contributor to The Cancer Genome Atlas.

The ideal UPCI director candidate will have an international reputation for innovative research as demonstrated by his/her record of peer-reviewed grant funding and publications, the ability to guide a large clinical network in providing exceptional care, a commitment to the education of the next generation of cancer researchers and clinicians, and broad leadership skills that have been demonstrated over time.

UPMC ranks among the top 12 hospital systems nationally (*U.S. News & World Report*); the University of Pittsburgh ranks fifth in total NIH funding. The University of Pittsburgh provides a highly competitive compensation and comprehensive benefits package. Further details about UPCI and the position are available at http://health.pitt.edu/sites/default/files/director_upci.html. Interested and qualified applicants should submit their CV and letter of interest to:

Search Committee, UPCI Director
c/o Office of Academic Affairs
University of Pittsburgh School of Medicine
Suite 401 Scaife Hall
3550 Terrace Street
Pittsburgh, PA 15261
or electronically to mmcdonald@hs.pitt.edu

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PHYSICIST

Weill Cornell Medicine - Qatar (WCM-Q)

In a pioneering international initiative, Cornell University and Weill Cornell Medicine (WCM) established Weill Cornell Medicine - Qatar (WCM-Q) through a unique partnership with the Qatar Foundation for Education, Science and Community Development. Operating in Doha, Qatar since 2002, WCM-Q offers a six-year integrated medical program including a two-year premedical curriculum, as well as a one-year foundation program to help prepare incoming students for medicine. WCM-Q seeks candidates for the position of

PHYSICIST

We are seeking a physicist who will have primary responsibility for the teaching of physics courses (including laboratories) to highly motivated students in their foundation and premedical studies. As appropriate, the successful candidate will also contribute to developing and team-teaching applications of physics in courses such as physiology, human structure and function, etc. In addition to the principal teaching obligation, the successful candidate will be expected to participate in student academic advising, committee work, and the broader academic and scholarly life of WCM-Q.

Eligible candidates will hold a Ph.D. degree in Physics or a closely related discipline. Preference will be given to candidates who demonstrate a record of excellence in and commitment to teaching and scholarship. Familiarity with the North American higher education system will be highly valued.

WCM-Q and WCM in New York share the same mission: to provide the finest education possible for its students, conduct research at the cutting edge of knowledge, improve health care, both now and for future generations, and provide the highest quality of care to the community. Full details regarding the WCM-Q program and facilities, including affiliations with ACGME-I accredited clinical sites, can be accessed at <http://qatar-weill.cornell.edu>.

A comprehensive and highly competitive salary and foreign-service benefits package, including fully furnished housing and other supplementary benefits, is provided. The appointment will be on a non-tenure-track and is normally for three years in the first instance, renewable by mutual agreement.

Qualified applicants are invited to submit a thoughtful letter of application outlining their interest in the position and how their skills and experience match WCM-Q's requirements, along with full curriculum vitae at <http://job.qatar-weill.cornell.edu>

Please note that due to the high volume of applications, only short-listed candidates will be contacted. The Search Committee will begin reviewing applications from the beginning of January 2017 and will continue until the position is filled.



**Weill Cornell
Medicine-Qatar**

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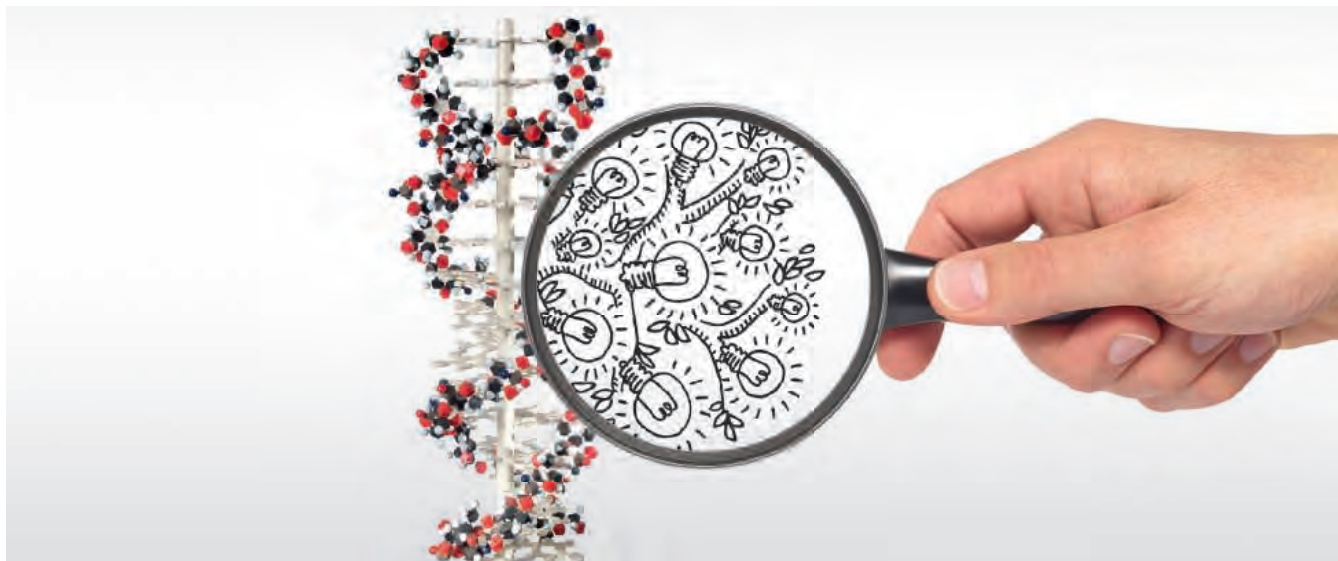
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**Professor/Associate Professor in
Prostate Cancer Research
Department of Cancer Biology
Lerner Research Institute (LRI)**

The Department of Cancer Biology is recruiting a prostate cancer researcher with strong translational interests at the professor or associate professor level. The position offers an exceptional opportunity to translate basic discoveries to the clinic by collaboration with outstanding clinical programs. The Prostate Cancer Research Center of Excellence, directed by Nima Sharifi and Eric Klein, is a multidisciplinary group of investigators who seek to define the underlying clinical and biochemical behavior of the lethal form of prostate cancer and identify new treatment strategies.

The Department has 12 primary faculty involved in basic and translational research, investigating cancers of blood, brain, breast, colon/rectum, as well as prostate. Prostate research includes (1) identifying prostate cancer metabolic phenotypes that confer treatment resistance; (2) novel therapeutic approaches involving poly(ADP)ribose modification and modulation of the DNA damage response; (3) determining functional impact of abnormal DNA methylation on prostate cancer biology; and (4) defining how molecular heterogeneity in androgen receptor transcriptional output translates into receptor action. Research across the department includes cell signaling, DNA damage and repair, tumor suppressor genes, novel targets, histone modification, cell cycle control, developmental therapeutics, drug resistance, invasion, metastasis, angiogenesis, tumor microenvironment, and innate defenses against viruses and cancer.

Applicants must have an MD, MD/PhD, or PhD; grant support; and an accomplished research program in prostate cancer. The successful applicant will receive generous start-up funds and joint appointment in Cleveland Clinic's Taussig Cancer Institute (part of the NCI-designated Case Comprehensive Cancer Center) and the Cleveland Clinic Lerner College of Medicine.

Candidates should e-mail a CV, summary of research interests, and 3 references to Loreen Burke, burkel2@ccf.org.

For further information see: <http://www.lerner.ccf.org/cancerbio/>

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**Tenure Track
Faculty Positions**

The Department of Cellular and Integrative Physiology in the School of Medicine at the University of Texas Health Science Center at San Antonio invites applications for tenure-track faculty positions at the Assistant, Associate and Professor level ranks. Individuals holding a Ph.D. and/or MD with an outstanding record of innovative research and academic performance, and with demonstrated expertise in areas of research that address developmental and functional relationships in the neurophysiological and neurovascular systems and associated pathologies are encouraged to apply. Successful candidates will receive highly competitive start-up packages and state-of-the-art laboratory space, and will be expected to establish vigorous externally funded independent research programs, provide exemplary mentorship, engage in productive scientific collaborations; and become members of our Integrated Biomedical Sciences Graduate Program to recruit and train graduate students. The positions will remain open until suitable candidates have been identified.

Applicants are encouraged to visit our website at <http://physiology.uthscsa.edu/> to learn about the department and the research of our current faculty and that of other neuroscientists at the Health Science Center at the Center for Biomedical Neuroscience website at <http://uthscsa.edu/cbn/index.asp>. A combined PDF file that includes curriculum vitae, a brief description of scientific achievements with current and future research interests (not to exceed 2 pages), and the names of three references should be emailed to Prof. Manzoor Bhat, Chairman, Department of Cellular and Integrative Physiology [email address: physiologysearch@uthscsa.edu].

The University of Texas Health Science Center at San Antonio is an Equal Employment Opportunity/Affirmative Action Employer including protected veterans and persons with disabilities.

All faculty appointments are designated as security sensitive positions.

FACULTY RECRUITMENT

**Mechanobiology Institute, Singapore
National University of Singapore**

The Mechanobiology Institute, Singapore is a multi-disciplinary institute committed to developing new paradigms for quantitative analysis of biological function. We are seeking outstanding candidates for tenure-track faculty positions at all levels with a background in molecular and cell biology, bacterial pathogenesis, correlative microscopies, biology-related engineering, or computational biology, and a commitment to collaborative interdisciplinary research. We offer an open lab environment and extensive central facilities support. Successful candidates will hold joint appointments through relevant departments at the National University of Singapore or other Singapore Universities.

Singapore fosters a rich and vibrant research environment. The National Research Foundation of Singapore offers generous research grants through the Singapore NRF Fellowships that can be awarded to outstanding young scientists of any nationality to support their independent research. (www.nrf.gov.sg)

Please submit your application along with curriculum vitae, full publication list, research plans and names of three external referees to :

Professor Michael Sheetz
Director
The Mechanobiology Institute, Singapore
National University of Singapore
T-Lab, 5A Engineering Drive 1
Singapore 117411
E-mail : mbihr@nus.edu.sg
Website : <http://mbi.nus.edu.sg>



**Associate Dean for Graduate Education
Division of Biology and Biomedical Sciences**

Washington University in St. Louis invites applications and nominations for the position of Associate Dean for Graduate Education in the Division of Biology and Biomedical Sciences (DBBS).

The new Associate Dean will advance the vision for DBBS, promote its mission, oversee its programs, direct its administration, and ensure that it continues its pioneering work. With new authority over and accountability for the Division, the Associate Dean will report to an Oversight Committee comprising the University's Provost, the Dean of the School of Medicine, and the Dean of the Faculty of Arts & Sciences. S/he will hold a tenured faculty appointment in a department affiliated with DBBS and will maintain active in research and teaching.

The first such program in the U.S., DBBS administers 12 interdepartmental graduate programs. Its 475 faculty have appointments in 37 departments across Arts & Sciences and the Schools of Medicine and Engineering & Applied Science. Some 650 students are pursuing PhD or MD/PhD degrees within these departments. For additional information, see: <http://dbbs.wustl.edu/>.

The successful candidate will possess a vision for and proven track record of accomplishment in graduate training and education in the basic sciences and will be qualified for a tenured faculty appointment.

Opus Partners is supporting this recruitment. Please send inquiries and nominations to Craig Smith (craig.smith@opuspartners.net) or Jennifer Romain (jennifer.romain@opuspartners.net). Candidates may also apply via: <https://facultyopportunities.wustl.edu/Posting/Detail/1010079>. Applications received by January 31st will receive full consideration.

Washington University in St. Louis is committed to the principles and practices of equal employment opportunity. It is the University's policy to recruit, hire, train, and promote persons in all job titles without regard to race, color, age, religion, sex, sexual orientation, gender identity or expression, national origin, protected veteran status, disability, or genetic information.

Chair, Department of Biostatistics and Computational Biology Dana-Farber Cancer Institute

Professor of Biostatistics Harvard T.H. Chan School of Public Health

The Dana-Farber Cancer Institute and the Harvard T.H. Chan School of Public Health are seeking a distinguished scientist to serve as chair of the Department of Biostatistics and Computational Biology at the Dana-Farber Cancer Institute. The successful candidate will also be appointed as a tenured professor in the Department of Biostatistics at the Harvard T.H. Chan School of Public Health and will provide leadership in the cancer training and research program in the department.

The Department of Biostatistics and Computational Biology at the Dana-Farber Cancer Institute is an active department of 21 faculty, 12 doctoral research scientists, 21 masters-level statisticians, and 12 bioinformatics analysts/engineers conducting wide-ranging methodological research in biostatistics and computational biology and collaborative research in cancer. The department is home to the statistical centers for the International Breast Cancer Study Group and the ECOG-ACRIN Cooperative Group, coordinates the Biostatistics Core Facility for the Dana-Farber/Harvard Cancer Center, and is the principal site of the Centers for Cancer Computational Biology, Functional Cancer Epigenetics, and Center for Cancer Evolution and cBio Center. The department is closely affiliated with the Department of Biostatistics at the Harvard T.H. Chan School of Public Health, where many of the department's faculty hold primary appointments and participate in the graduate training program.

The successful candidate will be a visionary leader, internationally recognized as a pre-eminent scientist with an established record of scholarship, ideally in the area of cancer research. Candidates should hold a doctoral degree in a relevant field.

Please submit a letter of application, including a statement of current and future research interests, a curriculum vitae, and sample publications, online at <http://academicpositions.harvard.edu/postings/7145> . It would be helpful if you would also provide the names of senior scholars likely to be most knowledgeable about your field and about your work in particular. Please contact facultyaffairs@hsph.harvard.edu with any questions.

Dana-Farber Cancer Institute and Harvard University seek to find, develop, promote, and retain the world's best scholars and are Affirmative Action/Equal Opportunity Employers. Applications from women and minority candidates are strongly encouraged.

The Department of Biostatistics and Computational Biology at Dana-Farber Cancer Institute provides a flexible working environment and provides a balance between work and life. Information on resources for career development and work/life balance at the Harvard Chan School can be found at: <https://hlc.harvard.edu/hlc-work-life-programs-at-a-glance/>.

We are an equal opportunity employer and all qualified applicants will receive consideration for employment without regard to race, color, religion, sex, sexual orientation, gender identity, national origin, disability status, protected veteran status, or any other characteristic protected by law.

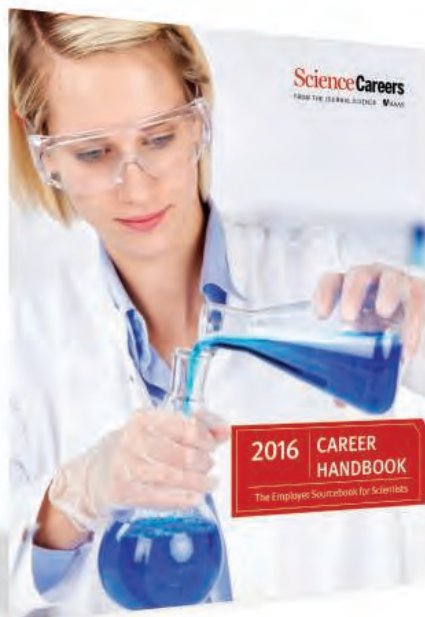
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Prof. Jan Kozłowski of Jagiellonian University in Kraków received the 2016 Prize in the life and earth sciences for formulation and experimental verification of a theory explaining the diversity of life strategies of organisms resulting from optimal allocation of resources.

Prof. Marek Samoć of Wrocław University of Science and Technology received the 2016 Prize in the chemical and material sciences for research on nanostructural materials for nonlinear optics.



Prof. Józef Spątek of Jagiellonian University in Kraków received the 2016 Prize in the mathematical, physical and engineering sciences for research on strongly correlated electron systems and, in particular, for derivation of the t-J model.

Prof. Bogdan Wojciszke of SWPS University of Science and Humanities, Sopot campus, received the 2016 Prize in the humanities and social sciences for developing a model of agency and communion as basic dimensions of social cognition.



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Director of a new research institute in Poland

The Foundation for Polish Science is offering > EUR 8 MLN to individual, senior scientists in the applied sciences to open their own autonomous research institutes in Poland. Funding is available until 2023 and applicants should be willing to spend at least 50% of their time on the project.

Requirements focus on the creation of a research agenda and strategic partnership with a research institution from outside Poland.

The research agenda should outline a clearly defined scientific or technological problem and an innovative method for solving it. The challenge should be important enough and the solution sufficiently cutting-edge to guarantee the IRAP centre a leading position worldwide.

The programme provides funding for renting space and equipment, however only very limited funding is available for new infrastructure. Therefore, partnership with a local institution may be required. The Foundation offers assistance with finding a suitable partner.

The project is funded within the framework of EU support for the International Research Agendas Programme (IRAP) and is one element of a multipronged strategy to create a dynamic, knowledge based economy in Poland with a strong scientific and R&D component.



APPLICATION
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Brigham and Women's Hospital
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We invite applications for a tenure-track Assistant Professor position; PhD. and/or M.D. will join pain and inflammation research mission of this department. Successful candidates will carry out research focused in Translational pain/inflammation, receptors or metabolomics in inflammation-resolution research. Basic and Translational research and mentoring are integral to these positions.

Qualified individuals with post-doctoral training and/or faculty experience should submit a cover letter, and curriculum vitae online at website: <https://research.bwhanesthesia.org/research-groups/cetri/serhan-lab/position>.

The names of three references and application should be addressed to Prof. Charles N. Serhan and James P. Rathmell, M.D., Search Committee Co-Chairs.

Deadline: March 1st, 2017

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香港中文大學
The Chinese University of Hong Kong

Dean of the Faculty of Science

Founded in 1963, The Chinese University of Hong Kong (<http://www.cuhk.edu.hk>) is a forward-looking and intellectually vigorous university with the mission to be a first-class comprehensive research university, regionally and internationally. With a team of over 3,000 full-time teaching and research staff, the University offers a broad spectrum of programmes up to the PhD level in various disciplines organized under eight Faculties (namely Arts, Business Administration, Education, Engineering, Law, Medicine, Science and Social Science). In 2015-16, the undergraduate and postgraduate enrolments in the University's publicly-funded programmes have reached 16,500 and 3,500 respectively.

The Faculty of Science (<http://www.cuhk.edu.hk/sci/>) comprises the Departments of Chemistry, Mathematics, Physics, and Statistics, and the School of Life Sciences, and also offers a number of multidisciplinary and interfaculty programmes. The Faculty has about 300 full-time teaching and research staff, over 2,700 undergraduate students and more than 500 postgraduate research students.

The University now invites applications and nominations of qualified candidates for the Deanship of the Faculty. The Dean will be a member of the University senior management team, reporting to the University Council via the Vice-Chancellor/President or the Provost. As the academic and executive head of the Faculty, the Dean will provide academic leadership and discharge administrative responsibilities in respect of academic, staff, resource (budget and space) as well as student matters. He/she will also actively engage in alumni and community relations and in extending networks.

Candidates should have an excellent academic standing appropriate for appointment at the level of a full Professor in the Faculty. They should have an appreciation of the breadth of research/educational developments in the relevant fields and the range of intellectual interests represented in the Faculty, demonstrated capability of academic leadership and strategic management in higher education institutions, a long-term vision for the development of the Faculty, and excellent interpersonal and communication skills.

Salary and fringe benefits for the post will be highly competitive, commensurate with qualifications and experience.

Please send applications/nominations under confidential cover to the Search Committee for the Dean of the Faculty of Science, c/o Secretary, Search Committee for the Appointment of the Dean of the Faculty of Science, Personnel Office, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong [fax: (852) 3942 0947; e-mail: SCDeanship-Sc@cuhk.edu.hk]. All applications/nominations will be treated in strict confidence. The University's Personal Information Collection Statement will be provided upon request.

Consideration of applications/nominations will continue until the post is filled. The University reserves the right to fill the post by invitation.



**UNIVERSITÉ
DE GENÈVE**
DIVISION DES
RESSOURCES HUMAINES

The Faculty of Science of the University of Geneva invites applications for the position of:

**ASSISTANT PROFESSOR
in MOLECULAR PLANT SCIENCES
(tenure track)**

JOB DESCRIPTION: The successful candidate is expected to develop an ambitious, independent research program using molecular tools to address fundamental questions in plant biology. The position comes with a competitive package, including substantial yearly core funding for research, which should be supplemented by acquiring external financing. State-of-the-art facilities are available allowing innovative research with potential for high impact to be realized.

The position is full time and includes 4 hours per week of teaching spread across different courses and seminars at the Bachelor and Master level, as well as overseeing and directing the research of undergraduate and graduate students.

The Department of Botany and Plant Biology at the University of Geneva offers a stimulating, highly dynamic scientific environment, renowned internationally for its research in the Molecular Plant Sciences. Geneva is a cosmopolitan city that offers numerous cultural and outdoor activities.

ACADEMIC TITLE AND EXPERIENCE REQUIRED:

- PhD degree.
- Publications in international journals.

STARTING DATE: September 2017 or a date to be agreed.

Applications (motivational cover letter, CV, list of publications, research and teaching statements and list of referees) must be posted online by **February 10th, 2017** on the Geneva University website: <https://jobs.unige.ch/> where this advert and the job description can be viewed.

No hard copies will be accepted.

More information can be found on our website: <http://biveg.unige.ch/en/>

The University of Geneva is an equal opportunity employer and encourages applications from female candidates.



UNIVERSITY of
ROCHESTER
MEDICAL CENTER

**The University of Rochester
Wilmot Cancer Institute**

Junior Faculty Positions

The *Wilmot Cancer Institute* at the University of Rochester Medical Center will recruit three tenure-track *Assistant Professors* with expertise in the broad area of *cancer biology*. The successful candidates will benefit from a multidisciplinary research community, a vibrant graduate program and state of the art infrastructure and core facilities at the University of Rochester. Competitive start-up packages are available. URMIC has a strong commitment to career development.

Qualified candidates holding a PhD and/or MD degree with an accomplished publication record who study basic or translational problems in cancer biology at the molecular, genomic or systems level are invited to apply. Scientists who use functional and/or computational genomics to conduct research are encouraged. Department affiliation will be determined according to the best fit.

The scientific interests pursued in the Wilmot Cancer Institute include cancer cell metabolism, cancer stem cell biology, RNA biology, signal transduction, systems biology, the tumor microenvironment and tumor immunology.

Chair of search committee: Hartmut Land, PhD. Candidates with a strong record of accomplishment should submit a CV, statement of research interests/plans, pdfs of two publications, and arrange to have three letters of recommendation sent to: Elva_Mikk@urmc.rochester.edu. Review of applications will start **January 15, 2017**.

The University of Rochester is an Equal Opportunity Employer and has a strong commitment to diversity and actively encourages applications from candidates from groups underrepresented in higher education.

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5. Watch one of our many webinars on different career topics such as job searching, networking, and more.
6. Download our career booklets, including Career Basics, Careers Beyond the Bench, and Developing Your Skills.
7. Complete an interactive, personalized career plan at “my IDP.”
8. Visit our Career Forum and get advice from career experts and your peers.
9. Research graduate program information and find a program right for you.
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Rescuing my time from science

It was a cold autumn day a couple years ago when I first felt that pursuing a Ph.D. was warping my sense of time. I had started working at 8:30 in the morning. By 8 in the evening, when almost everybody else had left, I was still in my office. I had so much work still to do that I reluctantly called my life partner to tell her that I would not be home for dinner. As I finally headed home around midnight, walking down dark, empty corridors and through the deserted university square, I realized that I was living in a sort of time warp—which was particularly ironic given that I was studying how daily activities shape our sense of time. Now, though, after years of allowing my work to take over my life, I'm reclaiming my power to decide how to spend my time.

When I started working toward my Ph.D., I never expected that it would take up so much of my time. But as I observed many fellow doctoral students and postdocs working long hours in the lab, I felt that, if I wanted to become a scientist, I needed to do the same. As I learned more about the increasing competitiveness of the academic job market and the importance of publishing frequently and well, I felt further pressure to spend nearly all my time at work. I was lucky that my supervisor did not explicitly demand extreme hours, as some lab heads do. Even so, I—like many other scientists—ignored the “normal” rhythms of work. I spent evenings and weekends studying the literature, designing experiments, collecting and analyzing data, writing papers, and replying to reviewers.

I'm proud of the work I've done, but I've paid a price for it. I sacrificed what I hold most dear: time for my life partner, my family, and my friends. On many occasions, I turned down invitations for social gatherings and special occasions because I had to work. I most regret missing my best friend's birthday celebration last year because the next day I had two deadlines, one for submitting a paper and the other for a review. The worst part is that I hadn't chosen to prioritize my job over my personal life; I just let it happen.

After completing my Ph.D. studies this past February, exhausted, I took a break for a few days before starting my postdoc. I finally thought with a clear head about my doctoral journey and about how I would like my life to look in the future. I realized that I could not continue working as I had. For me, the time for change has come. My loved ones deserve this, and I deserve it, too.

So, little by little, I am now consciously trying to bal-



“I am now consciously trying to balance my work and personal time.”

ance my work and personal time. I'm forcing myself to limit my job to five working days, only working during late night and weekend hours if it is strictly necessary, and I no longer feel guilty for taking time off. Thankfully, my adviser and my colleagues respect this choice. I'm learning to better organize my working hours by allocating time slots to specific tasks. I'm also starting to deal with deadlines more realistically, and I'm getting better at balancing my “no”s between my professional and social lives.

I do worry that these choices may put me at a disadvantage in the academic world. By aiming to work fewer than 50 hours a week, instead of my previous 70 or more, I will probably reduce my scientific production a little, even though I make a point of working efficiently and effectively. But I'm confident that my work's quality—which ultimately is more important than quantity—will remain high, or maybe even improve, especially in the long term. Regardless, this is the right choice for me.

It is all too easy to give too much power over important life decisions to our colleagues' behavior and the cultural expectations of academic research. For my part, I wish that I hadn't waited so long to take a step back from my work and reflect on where my time was going. But I'm heartened that I've done it now, and that I can finally spend my evenings with my life partner. And as my career moves forward, I will do my best to decide for myself how I want to spend my time. ■

Luca Rinaldi is a postdoctoral research fellow at the University of Milano-Bicocca in Italy. Send your career story to SciCareerEditor@aaas.org.